Rapid Paper**Fungal Trichothecenes Which Promote Callus Initiation from the Alfalfa Cotyledon**

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Three fungal trichothecenes, verrucarins A, roridin A and 8- β -hydroxyroridin E, were isolated as callus-initiating promoters from *Myrothecium* sp. 301. These trichothecenes promoted callus induction, synergistically coupled with a low concentration of 2,4-dichlorophenoxyacetic acid.

The use of plant tissue cultures for secondary products has been a central theme of recent research, and significant progress in the production of metabolites has been made by means of cell-selection and cell breeding techniques. It has been shown that useful bioactive compounds as well as pigments are only formed in the specific plant organs. For the production of secondary metabolites, callus cultures derived from a wide variety of plant organs or specific cell types are used. In general, the productivity of the secondary metabolites as well as the proliferation ability of the dedifferentiated tissues is highly dependent upon the source of explants. Therefore, the choice of explants will, to a large degree, be dictated by the choice of the research for this purpose. Attempts have been made to induce callus from a variety of plant specimens. However, callus initiation from the chosen organs with a variety of combinations of the known plant hormones may meet with difficulties, and not all plant species can be manipulated to induce callus as readily as the model systems of tobacco and carrot. In order to

overcome this problem, it is desirable to find a new callus-initiating agent which promotes callus induction from various organs of different plants. Among microbial metabolites, a preliminary screening program has been performed to find a new callus-initiating compound which promotes callus induction, synergistically coupled with auxins. For this purpose, alfalfa cotyledons were chosen as model explants, since the callus-inducing activity sharply responded to changes in the 2,4-D (2,4-dichlorophenoxyacetic acid) concentration in the medium.

It was found that a culture extract of *Myrothecium* sp. 301 promoted callus initiation from alfalfa cotyledons in the presence of a low concentration of an auxin, 2,4-D (0.1 μ g/ml), under which callus was not induced from the cotyledons without the extract.

Chromatographic separation of an extract of this culture on rice gave three active compounds (**1**, **2** and **3**). This paper deals with the isolation and structural elucidation of the three active compounds, as well as with their promoting activity toward alfalfa callus induction.

An acetone extract of the culture on rice of *Myrothecium* sp. 301 was concentrated, and the resultant aqueous concentrate was then partitioned against ethyl acetate. The ethyl acetate phase showed a significant callus-initiating activity together with 0.1 μ g/ml of 2,4-D. The active concentrate obtained after removing the ethyl acetate was chromatographed on a silica gel column to give three active fractions (20, 40 and 100% ethyl acetate in benzene). Recrystallization of the 20% and 40% ethyl acetate eluates yielded compounds **1** and **2**, respectively. The 100% ethyl acetate eluate was further purified with an ODS column to give compound **3**.

Compound **1** was isolated as colorless needles, mp 290°C, $[\alpha]_D^{20} + 244^\circ$ ($c=2.4$, CHCl_3), and characterized by an intensive fluorescence-quenching spot on a silica gel F₂₅₄ plate ($R_f=0.63$, 7:3 benzene-acetone) under a UV light and also by a brown spot when heating after spraying with vanillin- H_2SO_4 . The UV absorp-

tion band [$\lambda_{\max}$ (EtOH) nm (ϵ): 260 (18,000)] as well as the NMR splitting patterns of olefinic protons (δ 8.08: 1H, dd, $J=12, 16$ Hz; 6.67: 1H, dd, $J=11, 12$ Hz; 6.15: 1H, d, $J=11$ Hz; 6.04: 1H, d, $J=16$ Hz) suggested the presence of a muconic acid moiety. A characteristic pair of doublets (δ 2.80: 1H, d, $J=4.0$ Hz; 3.12: 1H, d, $J=4.0$ Hz) indicated the presence of an exocyclic epoxide. Consideration of these data as well as a literature survey of metabolites of the *Myrothecium* species suggested that this compound was structurally related to verrucarins. The MS spectrum (m/z 502, M^+) supported that this compound was verrucarin A. All signals in the ^1H -NMR spectrum (500 MHz, CDCl_3) were rationally assigned to all the protons of verrucarin A. Other physicochemical data (mp, $[\alpha]$, UV and IR) were consistent with those reported for verrucarin A.^{1~4)} Thus, **1** was identified as verrucarin A, which has been previously isolated from another *Myrothecium* species.^{1~4)}

Compound **2**, mp 192~194°C, $[\alpha]_{\text{D}}^{20} +127.6^\circ$ ($c=1.05$, CHCl_3), showed a very similar thin-layer chromatogram to that of **1** on a silica gel F₂₅₄ plate ($R_f=0.47$, 7:3 benzene–acetone). The spectral features of the UV [$\lambda_{\max}$ (EtOH) nm (ϵ): 263 (25,900)] and the ^1H -NMR data were almost identical with those of verrucarin A. However, an olefinic proton at δ 5.99 (1H dd, $J=5.7, 15.5$ Hz) assignable to H-7' was found to be coupled with a 1H double doublet at δ 3.56 ($J=5.7, 6.2$ Hz), indicating the presence of a 6-hydroxy-2,4-dienoic acid system. This suggested that **2** could be a roridin derivative, but not a verrucarin derivative.^{1,5~7)} All proton signals assignable to the trichothecene skeleton (H-2 to H-16) were nearly superimposable on those of **1**. Further analyses of the ^1H -NMR spectrum of acid portions as well as the MS spectrum (m/z 532, M^+) allowed us to identify this active compound **2** as roridin A, which has been previously isolated from the same genus.^{1,3,6)}

Compound **3** was isolated as a colorless oil, $[\alpha]_{\text{D}}^{20} -43.4^\circ$ ($c=1.6$, CHCl_3), and showed a

fluorescence-quenching spot on a silica gel F₂₅₄ plate under a UV light ($R_f=0.40$, 7:3 benzene–acetone; coloration toward vanillin– H_2SO_4 : brown). The UV absorption band [$\lambda_{\max}$ (EtOH) nm (ϵ): 262 (20,000)] as well as the proton splitting patterns of the dienoic acid moiety (H-7': δ 5.68, 1H, dd, $J=6.3, 16$ Hz; H-6': δ 3.71, 1H, d, $J=6.3$ Hz) characterized this compound as a close structural relative to the roridins. Analyses of the ^1H -NMR spectral data for the trichothecene skeleton and the acid moieties of the roridins suggested that **3** could be a roridin E derivative.^{7~10)} The MS spectrum (m/z 530, M^+ ; 512, $M^+ - \text{H}_2\text{O}$) also suggested that a hydroxyl was introduced into the trichothecene skeleton. The chemical shifts and splitting patterns of the protons on rings B and C in the trichothecene were nearly identical to those of roridin A. However, a 3H broad singlet (H-16) and a 1H broad doublet (H-10, $J=5.4$ Hz) were deshielded by 0.11~0.29 ppm in comparison with the corresponding signals of roridin E.^{9~11)} One H doublets at δ 4.13 ($J=5.7$ Hz) in **3** and at δ 5.19 ($J=5.1$ Hz) in the diacetyl derivative of **3** were ascribed to a carbinol proton, and the low chemical shifts indicated that a hydroxyl must be attached to C-8. This was also supported by the H–H long-range COSY experiments on the diacetate of **3**, that is, cross peaks were clearly seen between the C-16 methyl (δ 1.73, 3H, s) and C-8 methine (δ 5.19, 1H, br. d), and between the methine proton and a proton at C-7 (H-7 β : δ 2.27, dd, $J=5.1, 15.1$ Hz). The orientation of the C-8 hydroxyl was identified as β , based on a NOESY experiment on the diacetate of **3**, since NOEs were determined between H-4 (δ 6.10, dd, $J=4.2, 8.2$ Hz) and one of the methylene protons at C-15 (δ 4.50, 1H, d, $J=12.8$ Hz), and between H-8 (δ 5.19, d, $J=5.1$ Hz) and H-2' (δ 5.76, 1H, br. s). Thus, this new trichothecene (**3**) was determined as 8- β -hydroxyroridin E. In the NOESY experiments, intensive cross peaks were seen between one of H-15 (δ 4.08, 1H, d, $J=12.8$ Hz) and H-6' (δ 4.00, 1H, dd, $J=4.5, 5.0$ Hz), indicating that the stereochemistry of C-6' was of *R*-configuration, as in baccharin.¹²⁾ The stereo-

Verrucaric acid (1)	(2'S) (3'R) 12' -CH(OH)CH(CH ₃)CH ₂ CH ₂ OC(=O)- 6'	H
Roridin A (2)	(2'S) (3'R) (13'R) 14' -CH(OH)CH(CH ₃)CH ₂ CH ₂ OCHCH(OH)CH ₃ (6'R)	H
8-Hydroxyroridin E (3)	(2'E) (13'R) -CH=C(CH ₃)CH ₂ CH ₂ OCHCH(OH)CH ₃ (6'R)	OH
8-Hydroxyroridin E acetate (4)	(2'E) -CH=C(CH ₃)CH ₂ CH ₂ OCHCHCH ₃ (6'R)	OAc

chemistry of C-13' (*R*) was also determined by the presence of cross peaks between H-14' (δ 1.10, 3H, s) and H-14 (δ 0.77, 3H, s), and by the bond angle calculated from the coupling constant ($J=5.0$ Hz) between H-6' and H-13'. Biogenetic considerations of roridins in this species supports the stereochemistry for C-6' and C-13', since **2** and **3** were oxygenated products of roridin E (C-6', *R*; C-13', *R*) and could be biosynthesized from roridin E.

In the filtrate after the recrystallization of **1** and **2**, several minor metabolites with intensive UV-absorbing properties were detected by silica gel F₂₅₄ plates, and the partially purified fractions by a silica gel column also showed the same callus-initiating activity as these trichothecenes did. Further purification is now in progress.

Callus-initiating activity of the trichothecenes

Verrucaric acid (**1**) and roridin A (**2**) efficiently induced callus from the alfalfa cotyledon at concentrations of 2.0 μ g/ml and 0.25 μ g/ml,

Fig. 1. Callus Inducing Activity of the Trichothecenes toward Alfalfa Cotyledons.

The six cotyledons were grown at the optimal range of trichothecene concentrations, together with 0.1 μ g/ml of 2,4-D. (Roridin A showed phytotoxic activity at the concentrations of 1 ~ 4 μ g/ml.)

respectively, together with 0.1 μ g/ml of 2,4-D, when any signs of callus development were not seen (Fig. 1). Callus development started at the cotyledon in contact with the medium and grew rapidly. Ten days after treatment, the original greenish portion of the cotyledon dis-

appeared and gave well-developed clumps of callus (10-day old callus). The size and the weight of the clumps exceeded the levels of any dedifferentiated cotyledons cultured under the optimal 2,4-D concentration (1 $\mu\text{g/ml}$) for ten days. That is, the 10-day old callus cultured with 1 $\mu\text{g/ml}$ of 2,4-D and 1 $\mu\text{g/ml}$ of verrucarins A were 1.35 times the fresh weight of the clumps grown under the optimal 2,4-D concentration. After transplanting the 10-day old callus onto the phytohormone-free medium, this callus continuously developed and still maintained the potential to grow for the following several weeks. During this period, purple-colored cells predominantly developed. Soon after the 7-day old callus, which still partially possessed the original greenish tissue, was transferred onto the hormone-free medium, root formation started and well-developed roots were seen within the following ten days. This technique may be used to support effective organogenesis in tissue cultures of several economically important plant species.

It has not been clear what mode of operation these compounds have in a callus induction process when synergistically coupled with 2,4-D. Without any phytohormones, each of the three active compounds induced a small clump of callus at the cut end of the cotyledon. This clump grew slowly. This finding suggests that these compounds might stimulate IAA *de novo* synthesis or suppress the degradation of IAA, which was biosynthesized in the cut end when the cotyledon explant was prepared. The verrucarins and roridins are known as cytotoxic compounds against animal cells,^{8,13)} and they act as protein synthesis inhibitors.¹⁴⁾ Verrucarins A, roridin A and 8- β -hydroxyroridin E inhibited the alfalfa seed germination at concentrations of 50, 12.5 and 12.5 $\mu\text{g/ml}$, respectively. The germination inhibition should be due to the protein synthesis inhibitory activity of the trichothecenes. However, no direct correlation between the callus-initiating activity and the phytotoxicity was seen.

Experimental

1) *Preparation of alfalfa cotyledons.* Alfalfa seeds were sterilized by 70% ethanol for 5 min, following 10% hydrogen peroxide for 30 min. After being rinsed twice in sterile distilled water, the seeds were transferred onto sterilized 1% agar medium (10 ml) in a test tube (ϕ 25 mm $\times$ 10 cm), and then incubated for 7 days at 25°C in the light. Aseptically dissected cotyledons were prepared from the young seedlings.

2) *Screening method.* The culture broth (200 μl each of PSM) of a soil microorganism was added to the 2,4-D-containing Murashige-Skoog agar medium (2 ml; 2,4-D content, 0.1 $\mu\text{g/ml}$) in a test tube (ϕ 18 mm $\times$ 10 cm), and the medium was then sterilized by autoclaving. The cotyledons were transferred onto the medium and incubated for 10 days at 22°C in the light. The activity of the isolated compounds was also evaluated by the same method. The growth rate of the induced callus was determined as its increase in weight after a 10-day incubation.

3) *Extraction and isolation.* *Myrothecium* sp. 301, isolated from a soil sample, was inoculated on sterile steamed rice (5 kg), and then incubated for 30 days at 30°C in the static condition. The solid culture was soaked in 15 l of acetone for 2 days at room temperature, and then filtered. The filtrate was evaporated to give an aqueous crude concentrate, which was extracted twice with ethyl acetate (1 l $\times$ 2). The resulting ethyl acetate portion was dehydrated over anhydrous sodium sulfate and concentrated under reduced pressure to afford a crude extract (51 g). This extract was chromatographed on a silica gel column (Wako C-100, 500 g), eluting stepwise with 600 ml each of 10, 15, 20, 25, 30, 40, 50, 70 and 100% EtOAc in benzene. Recrystallization of the 20% EtOAc and 40% EtOAc eluates gave **1** (200 mg) and **2** (90 mg), respectively. The 100% EtOAc eluate (300 mg) was further purified with an ODS (Merck, RP-18, 30 g) column, which was eluted by a linear gradient system with H₂O (400 ml)–MeOH (400 ml) and 160 fractions were collected. Compound **3** (22 mg) was obtained as a colorless oil after concentrating fractions #83–86.

4) *Properties of the active compounds.* Compound **1**, mp 290°C (dec.); $[\alpha]_D^{20} + 243.9^\circ$ ($c = 2.4$, CHCl₃); EI-MS m/z (rel. int.): 502 [M⁺] (1.7), 249 (22.2), 213 (12.8), 148 (17.7), 131 (76.3); UV λ_{max} (EtOH) nm (ϵ): 260 (18,000); IR ν_{max} (KBr) cm⁻¹: 3430, 2970, 1720, 1635, 1270, 1190; NMR δ_{H} (500 MHz, CDCl₃): 0.85 (3H, s, H-14), 0.88 (3H, d, $J = 7.0$ Hz, H-12'), 1.75 (3H, s, H-16), 1.80 (1H, dt, $J = 3.0, 3.0, 12.0$ Hz, H-4'), 1.93 (1H, m, H-4'), 2.23 (1H, dd, $J = 8.0, 15.0$ Hz, H-3 α), 2.36 (1H, m, H-3'), 2.48 (1H, ddd, $J = 4.5, 5.5, 15.0$ Hz, H-3 β), 2.80 (1H, d, $J = 4.0$ Hz, H-13), 3.12 (1H, d, $J = 4.0$ Hz, H-13), 3.57 (1H, d, $J = 5.5$ Hz, H-11), 3.86 (1H, d, $J = 5.5$ Hz, H-2), 3.98 (1H, ddd, $J = 3.0, 11.0, 12.0$ Hz, H-5'), 4.15 (1H, br. s, H-2'), 4.21 (1H, d, $J =$

12.0 Hz, H-15), 4.51 (1H, ddd, $J=2.5, 5.5, 11.0$ Hz, H-5'), 4.80 (1H, d, $J=12.0$ Hz, H-15), 5.43 (1H, d, $J=5.5$ Hz, H-10), 5.81 (1H, dd, $J=4.5, 8.0$ Hz, H-4), 6.04 (1H, d, $J=16.0$ Hz, H-7'), 6.15 (1H, d, $J=11.0$ Hz, H-10'), 6.67 (1H, dd, $J=11.0, 12.0$ Hz, H-9'), 8.08 (1H, dd, $J=12.0, 16.0$ Hz, H-8').

Compound **2**, mp 192~194°C; $[\alpha]_D^{20} + 127.6^\circ$ ($c=1.05$, CHCl_3); EI-MS m/z (rel. int.): 532 [M^+] (0.7), 514 [$\text{M}^+ - 18$] (0.45), 488 (25.8), 358 (3.6), 249 (10.6); UV λ_{max} (EtOH) nm (ϵ): 263 (25,900); IR ν_{max} (KBr) cm^{-1} : 3450, 2960, 1750, 1710, 1640, 1600, 1415, 1180, 1080, 920; NMR δ_{H} (500 MHz, CDCl_3): 0.81 (3H, s, H-14), 1.08 (3H, d, $J=7.0$ Hz, H-12'), 1.19 (3H, d, $J=6.0$ Hz, H-14'), 1.73 (3H, s, H-16), 2.00 (1H, m, H-3'), 2.21 (1H, ddd, $J=4.5, 5.0, 15.0$ Hz, H-3 β), 2.45 (1H, dd, $J=8.0, 15.0$ Hz, H-3 α), 2.80 (1H, d, $J=4.0$ Hz, H-13), 3.12 (1H, d, $J=4.0$ Hz, H-13), 3.53 (2H, m, H-5'), 3.56 (1H, m, H-6'), 3.58 (1H, d, $J=5.5$ Hz, H-11), 3.65 (1H, m, H-13'), 3.85 (1H, d, $J=5.0$ Hz, H-2), 4.10 (1H, d, $J=3.0$ Hz, H-2'), 4.41 (1H, d, $J=12.0$ Hz, H-15), 4.45 (1H, d, $J=12.0$ Hz, H-15), 5.43 (1H, d, $J=5.5$ Hz, H-10), 5.78 (1H, dd, $J=4.5, 8.0$ Hz, H-4), 5.80 (1H, d, $J=11.0$ Hz, H-10'), 5.99 (1H, dd, $J=5.7, 15.5$ Hz, H-7'), 6.65 (1H, t, $J=11.0$ Hz, H-9'), 7.76 (1H, dd, $J=11.0, 15.5$ Hz, H-8').

Compound **3**, $[\alpha]_D^{20} - 43.4^\circ$ ($c=1.6$, CHCl_3); MS m/z (rel. int.): 530 [M^+] (2.7), 513 (2.34), 512 (6.10), 248 (12.8); UV λ_{max} (EtOH) nm (ϵ): 223 (28,200), 262 (20,000); IR ν_{max} (KBr) cm^{-1} : 3425, 2975, 2935, 1720, 1650, 1600, 1435, 1415, 1325, 1285, 1215, 1183, 1150, 1075, 1035, 1020, 965; NMR δ_{H} (500 MHz, CDCl_3): 0.81 (3H, s, H-14), 1.15 (3H, d, $J=5.9$ Hz, H-14'), 1.66 (1H, d, $J=14.5$ Hz, H-7), 1.84 (3H, s, H-16), 1.98 (1H, ddd, $J=4.0, 5.0, 15.2$ Hz, H-3 β), 2.20 (1H, m, H-4'), 2.20 (3H, s, H-12'), 2.26 (1H, m, H-4'), 2.37 (1H, dd, $J=5.7, 14.5$ Hz, H-7), 2.56 (1H, dd, $J=8.0, 15.2$ Hz, H-3 α), 2.84 (1H, d, $J=4.0$ Hz, H-13), 3.13 (1H, d, $J=4.0$ Hz, H-13), 3.45 (1H, m, H-5'), 3.70 (1H, dq, $J=5.0, 5.9$ Hz, H-13'), 3.71 (1H, dd, $J=5.0, 6.3$ Hz, H-6'), 3.80 (1H, m, H-5'), 3.82 (1H, d, $J=5.0$ Hz, H-2), 4.13 (1H, d, $J=5.7$ Hz, H-8), 4.13 (1H, d, $J=13.2$ Hz, H-15), 4.14 (1H, d, $J=5.4$ Hz, H-11), 4.41 (1H, d, $J=13.2$ Hz, H-15), 5.59 (1H, d, $J=5.4$ Hz, H-10), 5.68 (1H, dd, $J=6.3, 16.0$ Hz, H-7'), 5.80 (1H, s, H-2'), 5.82 (1H, d, $J=11.0$ Hz, H-10'), 6.39 (1H, dd, $J=4.0, 8.0$ Hz, H-4), 6.60 (1H, t, $J=11.0$ Hz, H-9'), 7.58 (1H, dd, $J=11.0, 16.0$ Hz, H-8').

5) *Preparation of the diacetate of 3*. A solution of 10 mg of 8- β -hydroxyrivoridin **3** in 0.4 ml of pyridine and 0.2 ml of acetic anhydride was allowed to stand at room temperature for 18 h. The solvent was removed *in vacuo* and the residue was subjected to silica gel column (Kieselgel 60, Merck; ϕ 6 mm $\times$ 35 cm) chromatography, using a linear gradient of benzene (100 ml) to benzene-EtOAc (80:20, 100 ml). 8- β -Hydroxyrivoridin **3** diacetate (**4**), which showed a fluorescence-quenching spot ($R_f=0.50$) on a silica gel F₂₅₄ plate under a UV light (benzene-EtOAc 7:3) was obtained as an oil from fraction #12~14. $[\alpha]_D^{20} - 30.7^\circ$ ($c=0.3$, CHCl_3); EI-MS m/z (rel. int.): 614

[M^+] (0.9), 571 (1.7), 570 [$\text{M}^+ - 44$] (5.13), 554 [$\text{M}^+ - 60$] (5.41), 247 (10.2); UV λ_{max} (EtOH) nm (ϵ): 220 (22,400), 255 (16,300); IR ν_{max} (KBr) cm^{-1} : 3500, 3000, 1745, 1730, 1650, 1630, 1450, 1380, 1250, 1190, 1160, 1085, 1010, 970; NMR δ_{H} (500 MHz, CDCl_3): 0.77 (3H, s, H-14), 1.10 (3H, d, $J=6.3$ Hz, H-14'), 1.73 (3H, s, H-16), 1.98 (1H, d, $J=15.1$ Hz, H-7 α), 1.99 (3H, s, 13'-acetyl), 2.04 (3H, s, 8-acetyl), 2.08 (1H, ddd, $J=4.2, 5.2, 15.3$ Hz, H-3 β), 2.23 (3H, br.s, H-12'), 2.27 (1H, dd, $J=5.1, 15.1$ Hz, H-7 β), 2.39 (2H, m, H-4'), 2.50 (1H, dd, $J=8.2, 15.3$ Hz, H-3 α), 2.82 (1H, d, $J=4.0$ Hz, H-13), 3.09 (1H, d, $J=4.0$ Hz, H-13), 3.70 (2H, m, H-5'), 3.81 (1H, d, $J=5.2$ Hz, H-2), 3.95 (1H, d, $J=5.9$ Hz, H-11), 4.00 (1H, dd, $J=4.5, 5.0$ Hz, H-6') 4.08 (1H, d, $J=12.8$ Hz, H-15), 4.50 (1H, d, $J=12.8$ Hz, H-15), 5.19 (1H, d, $J=5.1$ Hz, H-8), 5.10 (1H, dq, $J=5.0, 6.3$ Hz, H-13'), 5.76 (1H, Br.s, H-2'), 5.77 (1H, d, $J=11.2$ Hz, H-10'), 5.70 (1H, d, $J=5.9$ Hz, H-10), 5.84 (1H, dd, $J=4.5, 15.7$ Hz, H-7'), 6.10 (1H, dd, $J=4.2, 8.2$ Hz, H-4), 6.54 (1H, t, $J=11.2$ Hz, H-9'), 7.32 (1H, dd, $J=11.2, 15.7$ Hz, H-8').

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