Antimalarial Activity of Macrocyclic Trichothecenes Isolated from the Fungus *Myrothecium verrucaria*

Masahiko Isaka,* Juntira Punya, Yuwapin Lertwerawat, Morakot Tanticharoen, and Yodhathai Thebtaranonth

National Center for Genetic Engineering and Biotechnology (BIOTEC), Rama 6 Road, Bangkok 10400, Thailand

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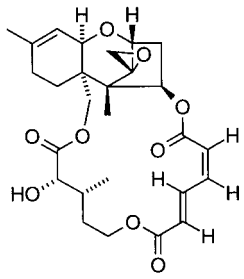
Bioassay-guided fractionation of an extract from the fungus *Myrothecium verrucaria* BCC 112 resulted in the first isolation of roridin E acetate (**5**) from nature together with four common macrocyclic trichothecene isomers (**1**−**4**). Trichothecenes **1**−**5**, while known as mycotoxins, were evaluated for their high antimalarial activity.

In an ongoing effort to identify novel naturally occurring antimalarial agents, we have screened a number of fungal cultures. Of these, a freeze-dried culture filtrate of *Myrothecium verrucaria* BCC 112 (Hypomycetes) showed high activity against *Plasmodium falciparum* (K1 strain) with an EC_{50} value of 190 ng/mL. We report herein the isolation and identification of antimalarial principles of this fungus.

Activity-guided fractionation of the crude AcOEt extract of culture filtrate using preparative HPLC (ODS column) resulted in the isolation of four major antimalarial constituents, identified as verrucarin A (**1**),[1] verrucarin J (**2**),[2] roridin A (**3**),[3,4] and roridin E (**4**).[5,6] These macrocyclic



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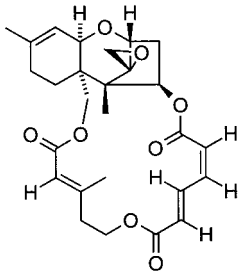

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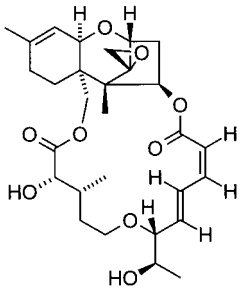

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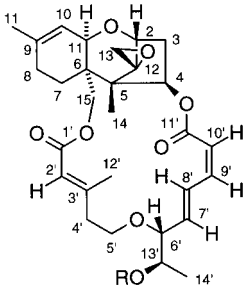

4: R = H
5: R = Ac

trichothecene mycotoxins[7,8] are known metabolites of *M. verrucaria*;[9−11] however, their activity against *P. falciparum* has not been evaluated. In addition to these major trichothecenes, several additional minor components were present in the AcOEt extract of the mycelium. Among these, the "highest-activity" HPLC fraction (EC_{50} 0.06 ng/mL) was isolated and purified. The ^1H NMR spectrum of this compound was similar to that of **4** except for an additional singlet peak (3H) at δ 2.05 and the downfield chemical shift of the one-proton multiplet due to H-13′ from ca. δ 3.6 in **4** to δ 5.12. Thus, the compound was suspected to be the acetate of **4**, whose structure is shown as **5**. Although roridin E acetate (**5**) has previously been semisynthesized by acetylation of roridin E (**4**) as a part of the structure elucidation of **4**,[5] there has never been a report of the isolation of this compound from natural resources. For structure confirmation, alcohol **4** was acetylated under the same conditions as reported.[5] Spectral data of the synthetic **5** were in all respects identical to that of naturally occurring **5** and to those reported in the literature. The isolated compound (**5**) was not an artifact due to acetylation of **4** during the course of mycelium extraction. This was confirmed by HPLC analysis where a peak corresponding to **5** was detected in the crude materials obtained from MeOH, acetone, and $CHCl_3$ extractions.

Antimalarial activities of the purified compounds **1**−**5** are given in Table 1. All five trichothecenes showed high in vitro activity against *P. falciparum* with EC_{50} values less than 1 ng/mL. Compound **5** is more potent than the well-known antimalarial drug, artemisinin, in our screening system.

Macrocyclic trichothecenes are known as mycotoxins,[12,13] and a variety of biological activities are reported for the common isomers such as **1** or **2**, which are particularly known for their high cytostatic activity.[14,15] For comparison with their antimalarial activities, the isolated compounds **1**−**5** were tested on cytotoxicity against certain cell lines (Table 1). All five compounds showed high cytotoxicity; however, the cytotoxicity was not in parallel with their in vitro antimalarial activity. For example, while the antimalarial activity of **5** was 15 times as strong as that of **1**, it was 12 times less cytotoxic against KB cell. In addition, the magnitude of antimalarial activity of **5** was much higher than its cytotoxicity as compared to other compounds in this series. It should also be noted that, while compounds **1**−**5** exhibited cytotoxicity in ng/mL concentrations, artemisinin showed no inhibition at 20 μg/mL. These observations suggest that it may be possible to find trichothecene derivatives with high antimalarial activity and much lower cytotoxicity.

Experimental Section

General Experimental Procedures.[16] Preparative HPLC was carried out on a Waters PrepLC 4000 system. Optical rotations were measured with a JASCO DIP-370 digital polarimeter. IR spectra were recorded on a Perkin-Elmer

* To whom correspondence should be addressed. Phone: (662) 644-8103. Fax: (662) 644-8107. E-mail: isaka@biotec.or.th.

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Table 1. Antimalarial Activity and Cytotoxicity of Trichothecenes from *Myrothecium Verrucaria*

compound	antimalarial activity (EC_{50}, ng/mL) *P. falciparum*	cytotoxicity (EC_{50}, ng/mL) KB cells[a]	BC1 cells[b]	vero cells[c]
verrucarin A (**1**)	0.90	1.2	4.2	1.1
verrucarin J (**2**)	0.20	3.9	14	1.2
roridin A (**3**)	0.31	1.0	5.0	1.7
roridin E (**4**)	0.15	0.5	0.7	0.4
roridin E acetate (**5**)	0.06	14	15	4.1
artemisinin	1.1	inactive[d]	inactive[d]	inactive[d]

[a] Human epidermoid carcinoma in the mouth (oral cavity). [b] Human breast cancer cells. [c] African green monkey kidney fibroblast. [d] Inactive (<50% inhibition at 20 μg/mL).

System 2000 spectrometer. ^{1}H NMR (400 MHz) and ^{13}C NMR (100 MHz) spectra were taken on a Bruker DRX400 spectrometer, with TMS (δ_H 0 ppm) and $CDCl_3$ (δ_C 77.1 ppm) as internal references, respectively.

Fungal Material. *M. verrucaria* BCC 112 was obtained from the BIOTEC culture collection, NSTDA, Thailand. Originally, it was collected from a soil sample at Kanchanaburi province, Thailand, and identified by Dr. Leka Manoch (personal communication).

Cultivation and Isolation. *M. verrucaria* was grown on PDA (potato dextrose agar) as an inoculum at 30 °C for 14 days. The well-grown culture was then cut into pieces of 0.5 × 0.5 cm blocks and inoculated into 250 mL of PDB (potato dextrose broth) in 28 1 L Erlenmeyer flasks. After static incubation at 30 °C for 14 days, the mycelia were separated from the culture broth by filtration, mechanically disrupted using a blender, and filtered by suction. The residue was redissolved in 80% aqueous MeOH (1 L) and washed with hexane (800 mL). The aqueous MeOH layer was concentrated, and the residual oil was dissolved in AcOEt (500 mL). The AcOEt solution was washed with H_2O (300 mL) and concentrated to obtain a brown oil (0.61 g). The crude extract was subjected to fractionation by preparative HPLC using a reversed-phase column (Prep Nova-Pak HR C_{18}, 6 μm, 40 × 100 mm) with MeCN/H_2O = 50:50 as eluent at a flow rate of 20 mL/min. A 6−8.5 min area containing two overlapping major peaks due to verrucarin A (**1**) and roridin A (**3**) was collected and further separated by silica gel column chromatography (Si 60H, 2−3% MeOH in CH_2Cl_2) to obtain pure **1** (7 mg) and **3** (10 mg). The HPLC peak at 16 min due to roridin E (**4**) was collected and purified by further HPLC (MeCN/THF/H_2O = 10:30:60 as eluent), followed by short silica gel column chromatography (2% MeOH in CH_2Cl_2) to obtain pure **4** (2 mg). Verrucarin J (**2**) was located in the HPLC 22 min peak, which was further purified by preparative HPLC with eluent MeCN/THF/H_2O = 20:30:50, followed by silica gel column chromatography (2% MeOH in CH_2Cl_2) to yield pure **2** (6 mg). The final peak on preparative HPLC, at 52 min due to roridin E acetate (**5**), was collected and further purified by silica gel column chromatography (1−2% MeOH in CH_2Cl_2) to provide **5** (1.4 mg).

Isolation from culture broth was also carried out. The culture filtrate (7 L) was separated into three portions. Each portion was extracted with an equal volume of AcOEt, and the combined organic layer was concentrated to obtain a yellow oil (0.71 g). This was subjected to fractionation by preparative HPLC, and further purification was carried out in the same manner as with the isolation from mycelium. Compounds **1**−**4** were isolated in the following yields: **1** (87 mg), **2** (5 mg), **3** (123 mg), and **4** (5 mg).

Roridin E acetate (5): colorless amorphous; $[\alpha]^{27}_D$ +43° (*c* 0.06, $CHCl_3$) [lit.[5] $[\alpha]^{23}_D$ +50° (*c* 0.85, $CHCl_3$)]; IR ($CHCl_3$) ν_{max} 3019, 1716 (C=O, ester), 1646, 1181, 1083, 969 cm^{-1}; ^{1}H NMR ($CDCl_3$, 400 MHz) δ 7.50 (1H, m, H-8′), 6.58 (1H, dd, *J* = 11.4, 11.3 Hz, H-9′), 6.13 (1H, dd, *J* = 8.1, 4.0 Hz, H-4), 5.90 (1H, s, H-2′), 5.88 (1H, m, H-7′), 5.75 (1H, d, *J* = 11.0 Hz, H-10′), 5.47 (1H, d, *J* = 5.3 Hz, H-10), 5.12 (1H, qd, *J* = 6.3, 4.9 Hz, H-13′), 4.35 (1H, d, *J* = 12.6 Hz, H-15a), 4.02 (1H, m, H-6′), 3.95 (1H, d, *J* = 12.6 Hz, H-15b), 3.86−3.84 (2H, m, H-2 and H-11), 3.74 (1H, m, H-5′a), 3.53 (1H, m, H-5′b), 3.13 (1H, d, *J* = 4.0 Hz, H-13a), 2.82 (1H, d, *J* = 4.0 Hz, H-13b), 2.53−2.45 (3H, m, H-3a and H-4′), 2.26 (3H, s, H-12′), 2.09 (1H, m, H-3a), 2.05 (3H, s, acetyl), 2.05−1.98 (3H, m, H-7a and H-8), 1.71 (1H, m, H-7b), 1.68 (3H, brs, H-16), 1.16 (3H, d, *J* = 6.5 Hz, H-14′), 0.80 (3H, s, H-14); ^{13}C NMR ($CDCl_3$, 100 MHz) δ 170.4 (CH_3CO), 166.6 (C-11′), 166.1 (C-1′), 159.1 (C-3′), 143.5 (C-9′), 140.3 (C-9), 137.5 (C-7′), 127.4 (C-8′), 119.0 (C-10), 117.9 (C-10′), 117.3 (C-2′), 79.3 (C-2), 79.2 (C-6′), 74.3 (C-4), 70.6 (C-13′), 69.0 (C-5′), 67.4 (C-11), 65.7 (C-12), 63.6 (C-15), 48.6 (C-5), 48.2 (C-13), 42.9 (C-6), 41.5 (C-4′), 35.7 (C-3), 27.8 (C-8), 23.3 (C-16), 21.4 (C-7), 21.3 (CH_3CO), 19.5 (C-12′), 14.8 (C-14′), 6.8 (C-14); FABMS *m/z* 557 $[M + H]^+$.

Acetylation of 4.[5] A solution of compound **4** (3.0 mg) in pyridine (0.2 mL) was treated with Ac_2O (0.1 mL) at room temperature for 12 h. The mixture was concentrated in vacuo, and the residual oil was purified by preparative HPLC (MeCN/H_2O = 70:30), followed by silica gel column chromatography (1−2% MeOH in CH_2Cl_2) to obtain the acetate **5** as a colorless amorphous solid (2.5 mg): $[\alpha]^{27}_D$ +48° (*c* 0.10, $CHCl_3$); *anal.* C 66.81%, H 7.39%, calcd for $C_{31}H_{40}O_9$, C 66.89%, H 7.24%. Spectral data (^{1}H and ^{13}C NMR, MS) were indistinguishable from those of naturally occurring **5**.

Antimalarial Activity. Continuous in vitro cultures of the asexual erythrocytic stage of *P. falciparum* (K1, multidrug rersistant strain) were maintained following the method of Trager and Jensen.[17] Quantitative assessment of antimalarial activity in vitro was determined using the microculture radioisotope technique based upon the method described by Desjardins.[18] Effective concentration (EC_{50}) represents the concentration that causes 50% reduction in parasite growth as indicated by the in vitro uptake of [^{3}H]hypoxanthine by *P. falciparum*.

Cytotoxicity Assay. Cytotoxicity of the purified compounds against human epidermoid carcinoma (KB), human breast cancer (BC1), and vero cell lines were tested using the protocol described by Likhitwitayawuid.[19]

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Supporting Information Available: Physical and spectroscopic data for compounds **1**−**4** (3 pages). Ordering information is given on any current masthead page.

References and Notes

(1) Gutzwiller, J.; Tamm, Ch. *Helv. Chim. Acta* **1965**, *48*, 157−176.
(2) Fets, E.; Bohner, B.; Tamm, Ch. *Helv. Chim. Acta* **1965**, *48*, 1669−1679.
(3) Bohrner, B.; Tamm, Ch. *Helv. Chim. Acta* **1966**, *49*, 2527−2546.
(4) Jarvis, B. B.; Midiwo, J. O.; Flippen-Anderson, J. L.; Mazzola, E. P. *J. Nat. Prod.* **1982**, *45*, 440−448.
(5) Traxler, P.; Zurcher, W.; Tamm, Ch. *Helv. Chim. Acta* **1970**, *53*, 2071−2085.
(6) Matsumoto, M.; Minato, H.; Tori, K.; Ueyama, M. *Tetrahedron Lett.* **1977**, 4093−4096.
(7) Review: Grove, J. F. *Nat. Prod. Rep.* **1993**, *10*, 429−448.
(8) Review: Tamm, Ch. *Fortschr. Chem. Org. Naturst.* **1974**, *31*, 63−117.
(9) Harri, E.; Loeffler, W.; Sigg, H. P.; Stoll, Ch.; Tamm, Ch. *Helv. Chim. Acta* **1962**, *45*, 839−853.

(10) Bohner, B.; Fetz, E.; Harri, E.; Sigg, H. P.; Stoll, Ch.; Tamm, Ch. *Helv. Chim. Acta* **1965**, *48*, 1079.
(11) Jarvis, B. B.; Stahly, G. P.; Pavanasasivam, G.; Midiwo, J. M.; DeSilva, T.; Holmlund, C. E.; Mazzola, E. P.; Geoghegan, R. F., Jr. *J. Org. Chem.* **1982**, *47*, 1117−1134.
(12) Bamburg, J. R.; Strong, F. M. In *Microbial Toxines*; Kadis, S., Ciegler, A., Ajl, S. J., Eds.; Academic Press: New York, 1971; Vol. 7, p 207.
(13) Cole, R. J.; Cox, R. H. In *Handbook of Toxic Fungal Metabolites*; Academic Press: New York, 1981; Chapter 5, pp 153−263.
(14) Jarvis, B. B.; Stahly, G. P.; Pavanasasviam, G. *J. Med. Chem.* **1980**, *23*, 1054−1058.
(15) Kupchan, S. M.; Streeman, D. R.; Jarbis, B. B.; Dailey, R. F. Jr.; Sneden, A. T. *J. Org. Chem.* **1977**, *42*, 4221−4225.
(16) Supporting Information is provided.
(17) Trager, W.; Jensen, J. B. *Science* **1976**, *193*, 673−675.
(18) Desjardins, R. E.; Canfield, C. J.; Haynes, J. D.; Chulay, J. D. *Antimicrob. Agents Chemother.* **1979**, *16*, 710−718.
(19) Likhitwitayawuid, K.; Angerholfer, C. K.; Cordell, G. A.; Pezzuto, J. M.; Ruangrungsi, N. *J. Nat. Prod.* **1993**, *56*, 30−38

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