14'-Hydroxymytoxin B and 16-Hydroxyroridin E, Two New Cytotoxic Trichothecenes from Myrothecium roridum

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14′-Hydroxymytoxin B and 16-Hydroxyroridin E, Two New Cytotoxic Trichothecenes from *Myrothecium roridum*

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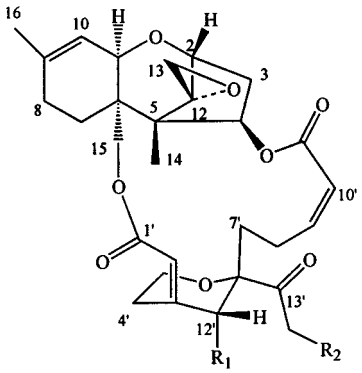
Two new trichothecenes, 14′-hydroxymytoxin B (**1**) and 16-hydroxyroridin E (**3**), were isolated from a fermentation extract of *Myrothecium roridum*. The structures of **1** and **3** were determined by spectral data interpretation. Both compounds showed potent cytotoxic activity against primary soft-tissue sarcoma cells.

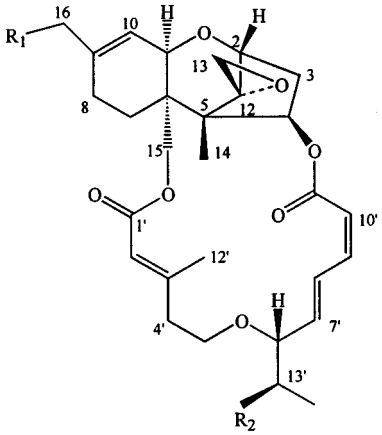
The trichothecenes are a group of sesquiterpenoids that have been isolated from various fungi, including species of *Cylindrocarpon*, *Fusarium*, *Myrothecium*, *Phomopsis*, *Stachybotrys*, *Trichothecium*, and *Verticimonosporium*, as well as a member of the plant genus *Baccharis*.[1] The common structural feature of this compound class is the 12,13-epoxytrichothec-9-ene ring system, and they can be divided into two main categories, namely, simple or nonmacrocyclic trichothecenes and macrocyclic trichothecenes. The nonmacrocyclic trichothecenes can be divided further into two subgroups based on the presence or absence of a carbonyl function at C-8. The macrocyclic trichothecenes are classified as roridins (mainly C_{29} compounds) or verrucarins (mainly C_{27} compounds). The trichothecenes have generated a great deal of interest due to their wide range of bioactivities, and in particular as antibacterial, antifungal, antimalarial, antitumor, and antiviral agents.[1−7]

In an ongoing effort to identify new cytotoxic substances from natural products, we have screened numerous microbial extracts in assays employing primary human tumor cells. In the course of this work, a fungal (*Myrothecium roridum*) extract was discovered that exhibited potent growth inhibitory activity against soft-tissue sarcoma cells. The initial fractionation of the ethyl acetate extract was performed by countercurrent partition chromatography (CPC) using a standard protocol.[8] Subsequently, the active CPC fractions were fractionated further by preparative HPLC, which afforded two new trichothecenes, 14′-hydroxymytoxin B (**1**, 2 mg) and 16-hydroxyroridin E (**3**, 1.5 mg), and six known trichothecenes, mytoxin B (**2**),[9] roridin E (**4**),[10] roridin D,[10] roridin L-2,[11] trichoverritone,[12] and verrucarin A.[10]

14′-Hydroxymytoxin B (**1**) was isolated as a white powder. The ion-spray mass spectrum showed the molecular weight to be 544 [545, $(M + H)^+$], with the molecular formula established as $C_{29}H_{37}O_{10}$ [$(M + H)^+$, 545.5970]. The presence of a characteristic pair of doublets in the ^{1}H NMR spectrum (δ 3.11, d, J = 4.0 Hz; δ 2.77, d, J = 4.0 Hz) indicated the presence of a methylene epoxide unit. These data and a consideration of the known secondary metabolites produced by *Myrothecium* spp. suggested that compound **1** is related structurally to the roridin group of macrocyclic trichothecenes.[13] The UV data (λ_{max} 229 nm),



1 R_1 = OH, R_2 = OH
2 R_1 = OH, R_2 = H
5 R_1 = OAc, R_2 = OAc



3 R_1 = OH, R_2 = OH
4 R_1 = H, R_2 = OH
6 R_1 = OAc, R_2 = OAc

along with the presence of two α,β-unsaturated lactone carbonyls (δ 166.5 and 166.3) and a ketone carbonyl carbon (δ 214.1) signal in the ^{13}C NMR spectrum, suggested that **1** could be a mytoxin derivative, rather than a roridin derivative.[13] Detailed analysis of the ^{1}H, ^{13}C, and COSY NMR data verified that **1** is a mytoxin B (**2**) derivative. Although the ^{1}H and ^{13}C NMR spectra of **1** (Table 1) were almost coincident with those of **2**, there were some differ-

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Table 1. ^{1}H and ^{13}C NMR Data for 14′-Hydroxymytoxin B (**1**) and 16-Hydroxyroridin E (**3**)[a,b]

	14′-hydroxymytoxin B (**1**)		16-hydroxyroridin E (**3**)	
position	δ_H	δ_C	δ_H	δ_C
2	3.82 d (5.4)	79.5	3.88 d (5.4)	79.8
3a	2.45 dd (15.6, 8.2)	35.4	2.56 dd (15.6, 8.2)	36.2
3b	2.14 ddd (15.6, 5.4, 3.6)		2.10 ddd (15.6, 5.4, 3.6)	
4	5.76 m	73.8	6.24 dd (8.2, 3.6)	74.5
5		50.0		48.9
6		43.3		43.6
7	1.98 m	20.9	2.00 m	21.3
8	2.10, 2.06 m	28.0	2.04, 1.78 m	23.5
9		140.8		143.4
10	5.41 d (4.3)	119.2	5.52 bd (5.0)	119.1
11	3.57 d (4.3)	68.1	3.99 d (5.3)	62.1
12		65.7		65.9
13a	3.11 d (4.0)	48.0	3.11 d (4.1)	48.5
13b	2.77 d (4.0)		2.88 d (4.1)	
14	0.77 s	8.1	0.83 s	7.1
15a	4.21 d (12.5)	64.8	4.35 d (12.5)	63.9
15b	3.92 d (12.5)		3.96 d (12.5)	
16	1.73 bs	23.7	4.07 bs	66.4
1′		166.3		164.8
2′	5.77 s	118.2	5.95 bs	117.4
3′		153.9		154.8
4a′	3.62 d (13.4)	26.0	2.54 m	41.6
4b′	2.70 m			
5′	4.05 m	63.7	3.45 m	70.3
6′		88.0	3.73 m	84.5
7′	1.74, 2.23 m	22.2	5.92 dd (15.6, 3.1)	138.5
8′	2.70, 2.06 m	28.6	7.54 dd (15.6, 11.4)	126.9
9′	6.37 ddd (11.7, 8.6, 6.7)	148.9	6.61 t (11.4)	144.2
10′	5.81 dd (11.7, 2.1)	121.3	5.78 d (11.4)	118.1
11′		166.5		166.2
12′	4.01 m	77.8	2.21	20.8
13′		214.1	3.66 m	71.1
14a′	4.67 d (20.2)	69.5	1.22 d (6.2)	18.7
14b′	4.47 d (20.2)			

[a] Spectra recorded in $CDCl_3$. [b] J values in Hz are shown in parentheses.

ences. Compound **2** showed a singlet for a methyl group at δ 2.30 (H_3-14′), but the corresponding signal was not observed in the ^{1}H NMR spectrum of **1**. Instead, two downfield-shifted methylene proton signals appeared at δ 4.67 and 4.47 (d, J_{AB} = 20.2 Hz). Similarly, the ^{13}C NMR signal for the C-14′ methyl group of **2** at δ 28.6[12] was missing in the ^{13}C NMR spectrum of **1**; instead, a signal for an oxygenated methylene group was observed at δ 69.5. The unusually large coupling constant (20.2 Hz) between these two methylene protons also suggested that they are adjacent to a ketone carbonyl carbon (proximity to π-bond or nonbonding electron pairs can cause greater geminal coupling).[14] In addition, upon acetylation, 14′-hydroxymytoxin B (**1**) formed a diacetate (**5**). These data confirmed the proposed structure for **1**, and it was evident that the C-14′ methyl group is oxidized to a primary alcohol in **1**. The ^{1}H−^{1}H connectivities were verified by analysis of the COSY spectrum, and ^{13}C NMR multiplicities and carbon and proton NMR connectivities were established by DEPT and HMQC experiments. The relative stereochemistry of 14′-hydroxymytoxin B (**1**) was assigned as being the same as in **2** on the basis of observed coupling constants and chemical shift comparison.[12]

16-Hydroxyroridin E (**3**) was isolated as a white powder. The ion-spray mass spectrum showed the molecular weight to be 530 [531, $(M + H)^+$], and a molecular formula of $C_{29}H_{38}O_9$ was established by HRFABMS. The UV absorption data (λ_{max} 218 and 258 nm) and the ^{1}H NMR spectrum revealed the presence of a dienoic ester moiety [δ 7.54 (dd, J = 15.6, 11.4 Hz), 6.61 (t, J = 11.4), 5.92 (dd, J = 15.9, 3.1 Hz), and 5.78 (d, J = 11.4)]. The ^{1}H and ^{13}C chemical shifts of **3** were almost identical to those of roridin E (**4**).[9] However, the ^{1}H NMR signal due to H-16 reported at δ 1.69 for roridin E was replaced by a two-proton singlet at δ 4.07 (Table 1). Therefore, it was apparent that the vinylic C-16 methyl group was oxidized to a primary alcohol in **3**. This was confirmed by a DEPT 135 spectrum, which showed only three methyl resonances (δ 20.8, 18.7, and 7.1) and one additional downfield methylene carbon resonance at δ 66.4. These data confirmed the proposed structure for **3** (16-hydroxyroridin E). The ^{1}H−^{1}H connectivities were again verified by analysis of a COSY spectrum and ^{13}C NMR multiplicities, and carbon and proton connectivities were established by DEPT and HMQC experiments.

The stereochemistry of **3** was assigned by comparison of its NMR data with those for roridin E (**4**) and isororidin E,[15] and it was found that the NMR data for 16-hydroxyroridin E (**3**) were very similar to those of roridin E (**4**).[9] Therefore, the stereochemistry of 16-hydroxyroridin E (**3**) was assigned as being the same as that of roridin E (**4**). This was confirmed by evaluating the ^{1}H NMR spectrum of 16-hydroxyroridin E acetate (**6**), which exhibited a five-line resonance at δ 5.14 (dq, $J_{6',13'}$ = 6.5, $J_{6',7'}$ 4.0 Hz, H-13′), characteristic for H-6′ in the roridin E *R*-epimer.[16]

All of the trichothecenes isolated were tested for inhibition of growth and viability of primary soft-tissue sarcoma cells. The IC_{50} values for 14′-hydroxymytoxin B (**1**), mytoxin B (**2**), 16-hydroxyroridin E (**3**), roridin E (**4**), verrucarin A, roridin D, roridin L-2, and trichoverritone were 1.3×10^{-9}, 8.4×10^{-10}, 4.6×10^{-8}, 7.6×10^{-10}, 2.9×10^{-10}, 9.5×10^{-10}, 3.0×10^{-8}, and 1.3×10^{-7} μM, respectively. Roridin L-2, 16-hydroxyroridin E (**3**), and trichoverritone were also tested for cytotoxic activity against high-grade leiomyosarcoma tumor cells and exhibited IC_{50} values of 1.8×10^{-8}, 8.5×10^{-8}, and 2.6×10^{-8} μM, respectively.

Experimental Section

General Experimental Procedures. Optical rotations were measured on a Perkin-Elmer 243B polarimeter in $CHCl_3$. UV spectra were run on a Perkin-Elmer Lambda 6 spectrometer. IR spectra were recorded on a Perkin-Elmer 1600 spectrometer. For NMR experiments (in $CDCl_3$) a Bruker DRX-500 spectrometer was used, operating at 500 MHz for ^{1}H and 125 MHz for ^{13}C NMR. Mass spectra were obtained on a PE Sciex API 150 MCA spectrometer interfaced with a Sciex ion-spray probe. Semipreparative HPLC was carried out on a Waters HPLC equipped with a Waters 600 system controller and a Waters 996 photodiode array detector.

Microorganism. Fungal strain no. 8420 was isolated from a soil sample obtained from Boston, MA, in August 1994. The culture was grown on yeast-malt extract agar in the dark at 25 °C. The spore mass was viscous and green when young, turning hard and black with age. Conspicuous sporodochia were formed on densely compacted, branching conidiophores, with the ultimate branches being phialides. Phialides were cylindrical, with conically tapering tips and undifferentiated collarettes. Conidia were olive-brown, single-celled, and cylindrical, with both ends rounded, 5.5−7.0 × 2.0 μm. On the basis of these characteristics, the fungus was identified as *Myrothecium roridum*.[17] The strain no. 8420 was deposited at the American Type Culture Collection under the ATCC no. MYA-2484.

Fermentation. The fermentation procedure utilized was a two-step process in which a suspension of spores and mycelium was inoculated into 250 mL flasks containing 25 mL of a nutrient seed medium having the following composition per liter: 20 g of glucose, 15 g of Pharmamedia (Trader's Protein), 5 g of yeast extract (Difco), 3 g of $(NH_4)_2SO_4$, 0.03 g of $ZnSO_4$, and 4 g of $CaCO_3$. After inoculation, the flasks were

incubated on a rotary shaker at 250 rpm and 28 °C for 2 days. Aliquots (1 mL) of the seed culture were then used to inoculate 100 × 250 mL flasks containing 30 mL of a production medium with the following composition per liter: 20 g of glucose, 50 g of sucrose, 20 g of Pharmamedia (Trader's Protein), 1 g of $NaNO_3$, 0.5 g of K_2HPO_4, 0.7 g of KCl, 1 g of L-histidine, and 0.014 g of $MgSO_4$. Following inoculation, the flasks of the production medium were incubated on a rotary shaker at 250 rpm and 28 °C for 7 days. The fermentation flasks were then harvested, and the fermentation mixture from each flask was pooled into a single vessel for extraction (~3 L).

Extraction and Isolation. The pooled fermentation mixture (broth and mycelium) was homogenized and then extracted with three equal volumes of EtOAc. The combined extracts were evaporated under reduced pressure to dryness to yield an oily residue (1.377 g). An aliquot (400 mg) of the crude extract was fractionated by dual-mode high-speed countercurrent chromatography (HSCCC, PC Inc., Potomac, MD). The solvent system and full chromatographic conditions were used as previously described.[8] The activity was concentrated into peaks eluting at 48−63 (fractions 17−21), 63−87 (fractions 22−29), 105−120 (fractions 36−40), and 156−168 (fractions 53−56) min. The active fractions were pooled and evaporated under reduced pressure to dryness.

Final purification was achieved using semipreparative HPLC. The mobile phase was pumped as a binary system at a rate of 10 mL/min and consisted of a 35 min linear gradient, starting with 70% H_2O−30% CH_3CN and ending with 100% CH_3CN. Roridin E (**4**, 4 mg) (t_R 14 min) and mytoxin B (**2**, 3 mg) (t_R 10.5 min) were isolated from the CPC fractions 17−21, verrucarin A (11 mg) (t_R 12.5 min) from fractions 22−29, 14′-hydroxymytoxin B (**1**, 2 mg) (t_R 22.5 min) and roridin D (2 mg) (t_R 23 min) from fractions 36−40, and 16-hydroxyroridin E (**2**, 1.5 mg) (t_R 7 min), roridin L-2 (2 mg) (t_R 6.5 min), and trichoverritone (1 mg) (t_R 11 min) from the CPC fractions 53−56.

14′-Hydroxymytoxin B (1): white powder; mp 182−184 °C, $[\alpha]^{25}$ +20° (*c* 0.002, $CHCl_3$); UV (MeOH) λ_{max} (log ϵ) 229 (2.45); IR ($CHCl_3$) ν_{max} 3500, 2925, 1715, 1653, 1640, 1355, 1179, 820 cm^{-1}; NMR data (Table 1); ion-spray MS (positive) *m*/*z* 545 $[M + H]^+$; HRFABMS *m*/*z* 545.5978 $[(M + H)^+$, calcd for $C_{29}H_{37}O_{10}$, 545.5970].

16-Hydroxyroridin E (3): white powder; mp 190−195 °C, $[\alpha]^{25}$ −29° (*c* 0.001, $CHCl_3$); UV (MeOH) λ_{max} (log ϵ) 218 (4.28), 258 (4.31); IR ($CHCl_3$) ν_{max} 3550, 3053, 2945, 1713, 1656, 1607, 1365, 1210, 1142, 815 cm^{-1}; NMR data (Table 1); ion-spray MS (positive) *m*/*z* 531 $[M + H]^+$; HRFABMS *m*/*z* 531.6143 $[(M + H)^+$, calcd for $C_{29}H_{39}O_9$, 531.6139].

General Procedure for Acetylation. Both trichothecenes **1** and **3** (1 mg each) were separately dissolved in ~100 μL each of pyridine and Ac_2O and allowed to stand at room temperature overnight. The solvent was removed by placing them under a N_2 stream.

14′-Hydroxymytoxin B acetate (5): amorphous solid; ^1H NMR (500 MHz, $CDCl_3$) δ 0.82 (3H, s, H-14), 1.75 (3H, bs, H-16), 1.70 (1H, m, H-7′a), 1.94 (1H, m, H-7), 2.03 (1H, m, H-8a), 2.08 (3H, s, acetyl), 2.14 (2H, m, H-8b and H-3b), 2.19 (3H, s, acetyl), 2.45 (1H, dd, *J* = 15.6, 8.2 Hz, H-3a), 2.52 (1H, m, H-7′b), 2.84 (1H, d, *J* = 4.0 Hz, H-13b), 3.15 (1H, d, *J* = 4.0 Hz, H-13a), 3.60 (1H, d, *J* = 4.3 Hz, H-11), 3.72 (1H, d, *J* = 13.2 Hz, H-4a), 3.84 (1H, d, *J* = 5.3 Hz, H-2), 4.01 (1H, d, *J* = 12.7 Hz, H-15a), 4.14 (1H, m, H-5′), 4.26 (1H, d, *J* = 12.7 Hz, H-15b), 5.03 (1H, d, *J* = 20.2 Hz, H-14′a), 5.18 (1H, d, *J* = 20.2 Hz, H-14′b), 5.45 (1H, bd, *J* = 4.3 Hz, H-10), 5.80 (1H, dd, *J* = 7.9, 3.6 Hz, H-4), 5.86 (1H, dd, *J* = 11.7, 2.1 Hz, H-10′), 6.06 (1H, bs, H-12′), 6.45 (1H, ddd, *J* = 11.7, 8.8, 6.7 Hz, H-9′); ion-spray MS (positive) *m*/*z* 629 $[M + H]^+$.

16-Hydroxyroridin E acetate (6): amorphous solid; ^1H NMR (500 MHz, $CDCl_3$) δ 0.84 (3H, s, H-14), 1.18 (3H, d, *J* = 6.1 Hz, H-14′), 2.07 (3H, s, acetyl), 2.09 (3H, s, acetyl), 2.23 (3H, s, H-12′), 2.85 (1H, d, *J* = 4.0 Hz, H-13b), 3.16 (1H, d, *J* = 4.0 Hz, H-13b), 3.55 (1H, m, H-5′b), 3.76 (1H, m, H-5′a), 3.77 (1H, m, H-6′), 3.99 (1H, d, *J* = 5.2 Hz, H-11), 3.88 (1H, d, *J* = 5.3 Hz, H-2), 3.95 (1H, d, *J* = 12.6 Hz, H-15b), 4.37 (1H, d, *J* = 12.6 Hz, H-15a), 5.14 (1H, dq, *J* = 6.5, 4.0 Hz, H-13′), 5.77 (1H, bd, *J* = 11.3 Hz, H-10′), 5.79 (1H, bd, *J* = 5.0 Hz, H-10), 5.86 (1H, s, H-2′), 5.95 (1H, dd, *J* = 15.6, 3.1 Hz, H-7′), 6.15 (1H, dd, *J* = 8.1, 3.8 Hz, H-4), 6.65 (1H, t, *J* = 11.3 Hz, H-9′), 7.52 (1H, dd, *J* = 15.6, 11.3 Hz, H-8′); ion-spray MS (positive) *m*/*z* 615 $[M + H]^+$.

Bioassay. A novel technique was used for screening for anticancer compounds, with tumor and nontumor tissues obtained from surgical resection on cancer patients. These tissues were typed by a panel of medical pathologists to determine the kind of cancer and the amount of tumor versus normal cells. Primary cell cultures were generated from both tumor and normal cells. Antitumor screening was conducted using these primary cell cultures. Cell preparations (both diseased and normal) that passed histological and cytological examination for diagnosis, grading, and cell purity were thawed at 37 °C and resuspended in tissue culture medium designed to maintain the cells during the incubation period. The live and dead cells were counted, and the tumor cells were diluted in culture medium to 1.0 × 10^3 cells/well. The cells were added to microtiter plates and incubated at 37 °C overnight with samples that were added at 1/10 the volume of the cell suspension. Alamar Blue (Accumed International, Westlake, OH) was then added to the cells at 1/10 the volume of the well, and the cells were further incubated at 37 °C for various times. Alamar Blue dye measures cellular redox reactions (i.e., cellular respiration), whereby a spectral shift occurs upon reduction of the dye (excitation 530 nm; emission 590 nm). The kinetics of cellular redox reactions were subsequently measured at 3 h and 3 days post-dye addition. These measurements, in comparison with control cells (untreated with compound) and media controls (test wells without cells), were used to determine the percent inhibition of the test compound, as well as their IC_{50} determinations.

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