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Four New Macrocyclic Trichothecenes from Two Strains of Marine-derived Fungi of the Genus *Myrothecium*

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Dedicated to the memory of Professor Kenneth L. Rinehart.

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Abstract Three new macrocyclic trichothecenes, named 12′-hydroxyroridin E (**1**), roridin Q (**2**), and 2′,3′-deoxyroritoxin D (**3**), were isolated from the marine-derived fungus *Myrothecium roridum* TUF 98F42, and a new macrocyclic trichothecene, named roridin R (**4**), was isolated from *Myrothecium* sp. TUF 02F6 together with roridins A and H and isororidin E. The structures of new compounds were determined on the basis of their spectral data. Compound **2** possessed a unique ether moiety at the 13′ position of **1**. Compound **4** was a 2′,3′-dihydro-2′-hydroxy derivative of roridin H. The IC_{50} values of compounds **1**, **2**, and **4** against the murine leukemia cell line L1210 were 0.19, 31.2, and 0.45 μM, respectively. Compound **3** showed antiyeast activity to *Saccharomyces cerevisiae* at 1 μg/disc (inhibition zone: 12.2 mm), which was about 10 time more active than roritoxin D (10.2 mm at 10 μg/disc).

Keywords *Myrothecium roridum*, marine-derived fungus, roridin Q, roridin R, macrocyclic trichothecene, cytotoxicity, L1210

Introduction

Macrocyclic trichothecenes are well known mycotoxins produced by several genera of fungi, such as *Baccharis*, *Cylindrocarpon*, *Myrothecium*, *Stachybotrys*, *Trichothecium*, and *Verticimonosporium* [1]. Recently, new macrocyclic trichothecenes and other compounds have been isolated from *Myrothecium* spp. possessing cytotoxic [2∼6], antimicrobial [3, 7], and tyrosinase inhibitory [8] activities.

In the course of our study on bioactive secondary metabolites of marine-derived fungi, we have reported a novel macrocyclic trichothecene derivative from *Myrothecium roridum* TUF 98F42 collected in Palau [2]. This compound had a double bond at the 12,13-position instead of the epoxide moiety of ordinary macrocyclic trichothecenes and was the second example of this structural modification. Further investigation on the secondary metabolites of the strain TUF 98F42 revealed the presence of three new macrocyclic trichothecenes named 12′-hydroxyroridin E (**1**), roridin Q (**2**), and 2′,3′-deoxyroritoxin D (**3**). We also found a new macrocyclic trichothecene, named roridin R (**4**), together with roridins

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A and H and isororidin E from *Myrothecium* sp. TUF 02F6 isolated from a marine sponge collected in Indonesia. We describe here the isolation, structure assignment, and cytotoxicity against the murine leukemia cell line L1210 of new compounds. Compound **2** had a unique ether moiety at the 13′ position of **1** and was more than 160-fold less active than **1** against L1210.

Materials and Methods

General Experimental Procedures

NMR spectra were measured on a JEOL AL-400, JNM A-500, or ECP-600 NMR spectrometer in $CDCl_3$ (δ_H 7.24, δ_C 77.0) or CD_3OD (δ_H 3.30, δ_C 49.0). Mass spectra were obtained by a JEOL HX-110 mass spectrometer (FAB mode). UV and IR spectra were recorded on HITACHI U-3000 and on JASCO A-102 spectrometers, respectively. Optical rotations were recorded with a JASCO DIP-370 digital polarimeter.

Producing Organisms

The fungus TUF 98F42 was isolated from a submerged woody material collected in Palau and identified as *M. roridum* [2]. *Myrothecium* sp. TUF 02F6 was isolated from an unidentified marine sponge collected in Manado, Indonesia. The fungi were cultured in 500-ml Erlenmeyer-flasks containing 150 ml of 1/2 PD medium (50% natural seawater) for about three weeks at 20°C as described in the previous papers [2, 9].

Extraction and Isolation

The culture broth of TUF 98F42 (900 ml) was filtered and extracted with EtOAc. The EtOAc extract was chromatographed on a silica gel column with EtOAc - hexane (gradient elution) and then with MeOH. The EtOAc - hexane (2 : 3) fraction was further separated by HPLC (ODS, 75% MeOH - H_2O) to give roridin E (7.5 mg), roritoxin D (1.7 mg), and **3** (3.8 mg). Compounds **1** (35.0 mg) and **2** (3.5 mg) were isolated from the MeOH eluate of the above silica gel column by HPLC (ODS, 65% MeOH - H_2O).

The culture broth of TUF 02F6 (1.2 liters) was filtered and extracted with hexane and EtOAc successively. The hexane extract was first separated by a silica gel column with EtOAc - hexane (gradient elution), and the 10% and 20% EtOAc fractions were purified by HPLC (ODS, 80% and 70% MeOH - H_2O) to give roridin A (13.7 mg), roridin H (7.9 mg), and isororidin E (6.2 mg). The EtOAc extract was chromatographed on a silica gel column with MeOH - $CHCl_3$ (gradient elution), and compound **4** (1.0 mg) was isolated from the 5% MeOH fraction by HPLC (ODS, 65% MeOH - H_2O) together with roridin A (26.0 mg).

12′-Hydroxyroridin E (1)

$[\alpha]_D^{25}$ +4.8° (*c* 4.0, MeOH). UV λ_{max}^{MeOH} nm (log ε) 257 (4.47). IR ν_{max} (KBr) cm^{-1} 3345, 1745, 1705, 1184, 1082. ^1H NMR (500 MHz, CD_3OD) and ^{13}C NMR (125 MHz, CD_3OD): see Table 1. ^{13}C NMR (100 MHz, $CDCl_3$) δ 173.8 (C-1′), 167.3 (C-3′), 166.3 (C-11′), 143.3 (C-9′), 140.5 (C-9), 138.8 (C-7′), 130.4 (C-8′), 118.8 (C-10), 118.6 (C-10′), 116.6 (C-2′), 85.3 (C-6′), 78.9 (C-2), 75.4 (C-4), 73.5 (C-12′), 69.7 (C-5′), 66.8 (C-11), 66.3 (C-13′), 65.6 (C-12), 62.5 (C-15), 48.9 (C-5), 48.0 (C-13), 44.3 (C-6), 36.2 (C-3), 29.3 (C-4′), 28.1 (C-8), 23.3 (C-16), 21.2 (C-7), 18.5 (C-14′), 6.7 (C-14). High-resolution (HR) FAB-MS *m/z* 531.2571 $[(M+H)^+$, Calcd for $C_{29}H_{39}O_9$, 531.2594].

Roridin Q (2)

$[\alpha]_D^{25}$ +2.4° (*c* 0.4, MeOH). UV λ_{max}^{MeOH} nm (log ε) 228 (5.53), 273 (4.72). IR ν_{max} (KBr) cm^{-1} 3434, 1740, 1705, 1142, 1075. ^1H NMR (600 MHz, CD_3OD) and ^{13}C NMR (150 MHz, CD_3OD): see Table 1. HRFAB-MS *m/z* 643.3114 $[(M+H)^+$, Calcd for $C_{35}H_{47}O_{11}$, 643.3118].

2′,3′-Deoxyroritoxin D (3)

^1H NMR (500 MHz, $CDCl_3$) δ 7.43 (1H, dd, *J*=16.5, 10.5 Hz, H-8′), 6.60 (1H, dd, *J*=11.0, 10.5 Hz, H-9′), 6.04 (1H, d, *J*=16.5 Hz, H-7′), 6.00 (1H, s, H-2′), 5.99 (1H, d, *J*=11.0 Hz, H-10′), 5.88 (1H, dd, *J*=8.5, 4.8 Hz, H-4), 5.43 (1H, d, *J*=5.4 Hz, H-10), 4.58 (1H, s, H-12′), 4.49 (1H, d, *J*=12.8 Hz, H-15a), 4.30 (1H, d, *J*=10.8 Hz, H-13′), 3.99 (1H, br d, *J*=15.0 Hz, H-4′a), 3.91 (1H, d, *J*=12.8 Hz, H-15b), 3.91 (1H, m, H-5′a), 3.83 (1H, d, *J*=5.2 Hz, H-2), 3.77 (1H, ddd, *J*=14.3, 12.2, 2.5 Hz, H-5′b), 3.57 (1H, d, *J*=5.4 Hz, H-11), 3.13 (1H, d, *J*=4.0 Hz, H-13a), 2.80 (1H, d, *J*=4.0 Hz, H-13b), 2.79 (1H, d, *J*=10.8 Hz, 13′-OH), 2.56 (1H, m, H-4′b), 2.44 (1H, dd, *J*=15.5, 8.5 Hz, H-3a), 2.20 (1H, ddd, *J*=15.5, 5.2, 4.8 Hz, H-3b), 2.03 (2H, m, H_2-8), 1.90 (2H, m, H_2-7), 1.71 (3H, s H_3-16), 0.79 (3H, s, H_3-14). ^{13}C NMR (125 MHz, $CDCl_3$) δ 173.4 (C-14′), 166.9 (C-11′), 165.1 (C-1′), 148.1 (C-3′), 141.0 (C-9′), 140.5 (C-9), 134.0 (C-8′), 128.0 (C-7′), 122.6 (C-10′), 122.4 (C-2′), 118.7 (C-10), 79.5 (C-12′), 79.1 (C-2), 79.0 (C-6′), 75.0 (C-13′), 74.4 (C-4), 68.0 (C-11), 65.4 (C-12), 64.5 (C-15), 60.9 (C-5′), 49.0 (C-5), 48.1 (C-13), 43.3 (C-6), 34.3 (C-3), 27.5 (C-8), 25.3 (C-4′), 23.3 (C-16), 20.2 (C-7), 7.5 (C-14). FAB-MS *m/z* 541 $[(M+H)^+]$.

Roridin R (4)

$[\alpha]_D^{25}$ +9.6° (*c* 0.05, $CHCl_3$). UV λ_{max}^{MeOH} nm (log ε) 263

(4.24). IR ν_{max} (KBr) cm^{-1} 1735, 1712, 1188, 1082. ^1H NMR (500 MHz, $CDCl_3$) δ 7.29 (1H, dd, J=15.8, 11.7 Hz, H-8′), 6.53 (1H, dd, J=11.7, 11.5 Hz, H-9′), 6.09 (1H, dd, J=15.8, 3.5 Hz, H-7′), 5.87 (1H, d, J=11.5 Hz, H-10′), 5.71 (1H, dd, J=8.0, 4.0 Hz, H-4), 5.40 (1H, d, J=4.9 Hz, H-10), 5.31 (1H, t, J=3.4 Hz, H-5′), 4.50 (1H, d, J=12.3 Hz, H-15a), 4.35 (1H, d, J=12.3 Hz, H-15b), 4.15 (1H, m, H-2′), 4.12 (1H, m, H-6′), 3.84 (1H, m, H-13′), 3.83 (1H, m, H-2), 3.56 (1H, d, J=5.2 Hz, H-11), 2.45 (2H, m, H-3a and H-3′), 2.19 (1H, m, H-3b), 1.98 (1H, m, H-8a), 1.96 (1H, m, H-4′a), 1.88 (1H, m, H-7a), 1.87 (1H, m, H-8b), 1.86 (1H, m, H-4′b), 1.75 (1H, m, H-7b), 1.72 (3H, s, H_3-16), 1.27 (3H, d, J=6.3 Hz, H_3-14′), 1.00 (3H, d, J=6.8 Hz, H_3-12′), 0.88 (3H, s, H_3-14). ^{13}C NMR (125 MHz, $CDCl_3$) δ 174.8 (C-1′), 167.1 (C-11′), 141.0 (C-9′), 140.7 (C-9), 135.9 (C-7′), 126.7 (C-8′), 120.1 (C-10′), 118.1 (C-10), 102.2 (C-5′), 81.4 (C-6′), 78.9 (C-2), 78.2 (C-13′), 75.4 (C-4), 74.8 (C-2′), 67.0 (C-11), 65.2 (C-12), 64.1 (C-15), 49.2 (C-5), 47.8 (C-13), 43.9 (C-6), 35.8 (C-4′), 35.0 (C-3), 32.5 (C-3′), 27.6 (C-8), 23.3 (C-16), 20.2 (C-7), 18.1 (C-14′), 13.2 (C-12′), 7.5 (C-14). HRFAB-MS *m/z* 531.2603 $[(M+H)^+$, Calcd for $C_{29}H_{39}O_9$, 531.2594].

Cytotoxicity

The growth inhibitory activity of compounds and fractions against L1210 cells was tested in 96-well plastic plates and the vital cells were detected by the XTT method. Samples were dissolved in MeOH and 10 μl of each sample solution was poured in a well and the solvent evaporated in a clean bench. The suspension of L1210 cells (1×10^5 cells/ml) was added into each well and the number of vital cells in the sample wells after 72 hours was compared with those in the control (MeOH) wells.

Results and Discussion

M. roridum TUF 98F42 produced 12′,13′-deoxyroridin E, roridin E, and verrucarins A and J [2]. The new fermentation of this strain gave three new compounds, named 12′-hydroxyroridin E (**1**), roridin Q (**2**), and 2′,3′-deoxyroritoxin D (**3**), and the known roritoxin D. *Myrothecium* sp. TUF 02F6 isolated from an unidentified marine sponge collected in Manado, Indonesia, produced a new compound, named roridin R (**4**), together with roridins A and H and isororidin E. The structures of known compounds were assigned on the basis of their spectral data and comparison with the reported values for roritoxin D [10], roridins A and H [1], and isororidin E [1]. The ^1H and ^{13}C NMR signals of purified compounds were assigned by ^1H-^1H COSY, HMQC, and HMBC spectra.

12′-Hydroxyroridin E (**1**) was isolated as a white powder. The molecular weight (530) and formula ($C_{29}H_{38}O_9$) were determined from the HRFAB-MS and

Table 1 ^1H and ^{13}C NMR data for compounds **1** and **2** in CD_3OD

C#	**1**[a]		**2**[b]	
	^{13}C (δ)	^1H (δ, mult, J in Hz)	^{13}C (δ)	^1H (δ, mult, J in Hz)
2	80.3	3.75, d, 5.5	80.4	3.75, m
3	37.8	2.53, dd, 15.7, 7.5	37.5	2.55, dd, 15.7, 7.5
		1.92, m		1.97, m
4	77.0	5.97, m	76.6	5.87, dd, 8.0, 3.3
5	50.1		49.3	
6	45.7		44.7	
7	21.9	1.89, m	22.2	1.98, m
		1.75, m		1.82, m
8	29.1	2.11, m	29.0	1.99, m (2H)
		1.98, m		
9	141.1		141.9	
10	119.6	5.40, d, 5.5	119.6	5.41, bs
11	68.5	3.76, m	68.3	3.83, m
12	66.8		66.6	
13	48.9	3.09, d, 3.8	48.6	3.10, d, 3.8
		2.86, d, 3.8		2.88, d, 3.8
14	7.7	0.87, s (3H)	7.6	0.80, s (3H)
15	62.3	3.83, d, 12.4	63.9	4.30, d, 12.4
		3.50, d, 12.4		4.04, d, 12.4
16	23.4	1.71, s (3H)	23.2	1.71, s (3H)
1′	176.7		176.1	
2′	116.6	5.95, s	116.8	5.96, s
3′	171.6		171.8	
4′	30.2	2.76, m (2H)	30.2	2.76, m (2H)
5′	67.7	3.76, m	67.8	3.74, m
		3.63, m		3.61, m
6′	86.2	3.74, m	86.3	3.73, m
7′	141.6	5.99, dd, 15.4, 7.5	141.3	5.98, dd, 15.4, 7.2
8′	131.3	7.62, dd, 15.4, 11.4	131.4	7.61, dd, 15.4, 11.3
9′	144.6	6.72, dd, 11.4, 11.4	144.9	6.71, dd, 11.3, 11.3
10′	119.2	5.70, d, 11.4	117.7	5.69, d, 11.3
11′	167.2		167.3	
12′	75.4	4.56, s (2H)	75.5	4.92, s (2H)
13′	70.4	3.74, m	70.5	3.73, m
14′	19.2	1.10, d, 6.0 (3H)	19.1	1.09, d, 6.0 (3H)
1″			167.6	
2″			119.1	5.75, s
3″			159.4	
4″			44.7	2.37, t, 6.3 (2H)
5″			60.6	3.70, m (2H)
6″			19.0	2.17, s (3H)

[a] ^1H: 500 MHz, ^{13}C: 125 MHz, [b] ^1H: 600 MHz, ^{13}C: 150 MHz.

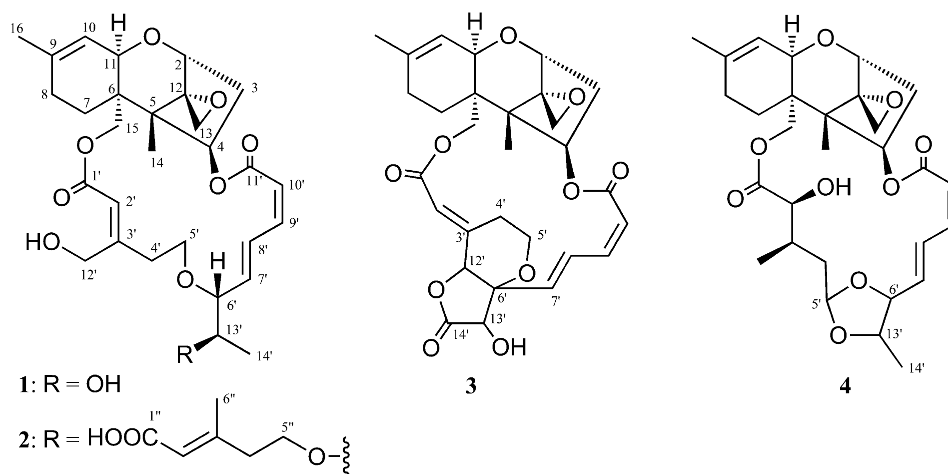

Fig. 1 Structures of compounds **1**~**4**.

NMR data. The ^{1}H and ^{13}C NMR spectra of **1** were similar to those of roridin E, except for the presence of signals (δ_H 4.56 (2H, s) and δ_C 75.4 in CD_3OD) ascribable to a hydroxymethyl group and the absence of signals at δ_H 2.25 (3H, s) and δ_C 20.3 in $CDCl_3$ observed in the NMR spectra of roridin E due to the 12′ methyl group. HMBC correlations were detected from H_2-12′ (δ 4.56) to C-1′ (δ 176.7), C-2′ (δ 116.6), and C-3′ (δ 171.6). Therefore, the structure of **1** was deduced to be the 12′-hydroxy derivative of roridin E.

The stereochemistry of **1**, except for the 6′ and 13′ positions, was assigned by the comparison of ^{13}C NMR data for **1** measured in $CDCl_3$ with those for roridin E isolated from the same strain (TUF 98F42). ^{13}C NMR data for **1** at C-5′ to C-8′, C-13′, and C-14′ observed in $CDCl_3$ were compared with those for roridin E (6′*R*,13′*R*), isororidin E (6′*S*,13′*S*), epiroridin E (6′*R*,13′*S*), and epiisororidin E (6′*S*,13′*R*) to determine the stereochemistries at the 6′ and 13′ positions [11], and the chemical shifts of the (6′*R*,13′*R*)-isomer (roridin E) were very similar to those of **1**. Thus the structure of **1** was assigned as shown in Fig. 1.

Roridin Q (**2**) was obtained as a white powder. The ^{1}H and ^{13}C NMR spectra of **2** contained very similar signals to those of **1** along with additional signals at δ_H 5.75 (1H, s), 3.70 (2H, m), 2.37 (2H, t, J=6.3 Hz), and 2.17 (3H, s) and at δ_C 167.6, 159.4, 119.1, 60.6, 44.7, and 19.0 (Table 1). The differences in NMR data between **2** and **1** were explained by the differences in the molecular weights and formulas (112, $C_6H_8O_2$) between **2** (642, $C_{35}H_{46}O_{11}$) and **1**. Key correlations were detected from H-6″ to C-1″, C-2″, C-3″, and C-4″ and from H-5″ to C-3″ and C-13′ in the HMBC spectrum of **2**. Therefore, the additional moiety was assigned as 3-methyl-5-oxy-2-pentenoic acid attached to the 13′ position through the ether linkage (Fig. 1). The carbon chain of this moiety was the same as that of the 1′~5′ and 12′ unit.

2′,3′-Deoxyroritoxin D (**3**) was isolated as a white powder. The molecular weight was determined as 540 from the FAB mass spectrum of **3**, and ^{1}H and ^{13}C NMR data for **3** suggested the molecular formula of $C_{29}H_{32}O_{10}$. The ^{1}H and ^{13}C NMR spectra of **3** resembled those of roritoxin D isolated from the same strain (TUF 02F6), except for the presence of a double bond (δ_H 6.00 (1H, s), δ_C 122.4 and 148.1) instead of the epoxide moiety at the 2′,3′-position in roritoxin D (δ_H 3.36 (1H, s), δ_C 57.4 and 60.6). HMBC correlations were observed from H-2′ to C-1′, C-3′, C-4′, and C-12′. Therefore, the structure of **3** was assigned as the 2′,3′-deoxy derivative of roritoxin D (Fig. 1). The stereochemistries at the 6′, 12′, and 13′ positions were not determined.

Roridin R (**4**) was obtained as a white powder. The molecular weight (530) and formula ($C_{29}H_{38}O_9$) were deduced from the HRFAB-MS and NMR data. The ^{1}H and ^{13}C NMR spectra of **4** revealed the presence of the same trichothecene moiety (C-2~C-16) as that in **1**~**3**. ^{1}H and ^{13}C NMR data for **4** due to the 6′ to 11′, 13′, and 14′ positions were very similar to those for roridin H isolated from the same strain (TUF 02F6). The characteristic anomeric carbon signal (C-5′) was shown at δ 102.2 in the ^{13}C NMR spectrum of **4**. The differences in NMR data between **4** and roridin H were detected at the partial structure of the 1′~5′ and 12′ unit. Signals ascribable to a hydroxymethine (δ_H 4.15 (1H, m), δ_C 74.8) and a CH-CH_3 unit (δ_H 2.45 (1H, m) and 1.00 (3H, d, J=6.8 Hz), δ_C 32.5 and 13.2) were observed in the NMR spectra of **4** instead of the olefinic signals due to the 2′, 3′ and 12′ positions in those of roridin H. The partial structure of the 1′~5′ and

12′ unit was elucidated by the ^{1}H-^{1}H COSY and HMBC spectra, and an HMBC correlation was detected from H-13′ (δ 3.84) to C-5′ (δ 102.2). The stereochemistries at the 2′ and 3′ positions were assigned by the comparison of ^{13}C NMR data for **4** with those for the 2′,3′-dihydro-2′-hydroxy compounds roridin A and verrucarins A and K [1], but the 5′, 6′, and 13′ positions were not determined. Thus the structure of **4** was assigned as shown in Fig. 1.

Compounds **1**, **2**, and **4** were tested for growth inhibitory activity against L1210. The IC_{50} values of **1**, **2**, and **4** were 0.19, 31.2, and 0.45 μM, respectively. In the same experiment, roridins A, E, and H showed IC_{50} values of 0.11, 0.11, and 0.12 μM, respectively. Cytotoxicity of **3** could not be determined because the compound had decomposed after the structure elucidation. Compound **3** inhibited the growth of *Saccharomyces cerevisiae* IAM 14383T at 1 μg/disc (inhibition zone: 12.2 mm), which was about 10 time more active than roritoxin D (2′,3′-epoxy derivative, 10.2 mm at 10 μg/disc).

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