Bioactive Trichothecenes Produced by the *Myrothecium* sp. QB-1

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Three new simple trichothecenes, 15-acetyltrichoverrol B (**3**), 13′-acetyltrichoverrin B (**5**), and 6′-dehydroxytrichoverrin B (**6**), along with five known trichothecenes trichodermadienediol B (**1**), trichoverrol B (**2**), trichoverrin B (**4**), and roridins A (**7**) and D (**8**), have been isolated from the liquid culture of *Myrothecium roridum* (strain no. QB-1). The structures of the new compounds were established by comprehensive analysis of 1D- and 2D-NMR data. All the compounds were evaluated for antifungal activity, only compounds **7** and **8** showed significant antifungal activity against the tested fungi (*MIC* ranged from 10 to 5 μg/ml).

Introduction. – Trichothecenes are a family of sesquiterpenols free of or with acyl residue(s) produced by fungi. The trichothecene group of terpenoid antibiotics may be classed into two distinct types: simple and macrocyclic [1], sharing a common biosynthetic path. Among this type of secondary metabolites, a number of them have been demonstrated to play an important role in cancer prevention [2], immunomodulation [3], plant tissue induction [4], phytotoxicity [5], fungus suppression [6], malaria management [7], biocontrol agent [8], and mammalian intoxications [9]. Hence, trichothecenes have become a common research topic in many fields such as agriculture, medicine, food safety, animal breeding, toxicology, and even biochemical antiterrorism, and attracted much more public attention.

In our previous characterization of biologically potent and/or chemically novel metabolites produced by fungi harboring in healthy plant tissues, we have reported three new cytotoxic 10,13-cyclotrichothecane-derived macrolides, myrothecines A – C, characterized from the extracts of two *M. roridum* strains, IFB-E009 and IFB-E012, isolated as endophytic fungi found on the traditional Chinese medicinal plants *Trachelospermum jasminoides* and *Artemisia annua*, respectively [10]. Here, we focus on a strain of *M. roridum* (Strain no. QB-1), separated from *Raphanus sativus* L. collected in October 2013, from the suburb of Jurong in Jiangsu Province (P. R. China). The fungus was fermented in liquid medium. Fractionation of the AcOEt extract afforded three new simple trichothecenes, 15-acetyltrichoverrol B (**3**), 13′-acetyltrichoverrin B (**5**), and 6′-dehydroxytrichoverrin B (**6**), together with five known trichothecenes, trichodermadienediol B (**1**), trichoverrol B (**2**), trichoverrin B (**4**) [11], and roridins A (**7**) and D (**8**; *Fig. 1*) [12]. All the compounds were evaluated for antifungal activity, compounds **7** and **8** showed significant antifungal activity against the tested fungal (*MIC* ranged from 10 to 5 μg/ml). Herein, we report the isolation, structure elucidation, and biological activity of these trichothecenes.

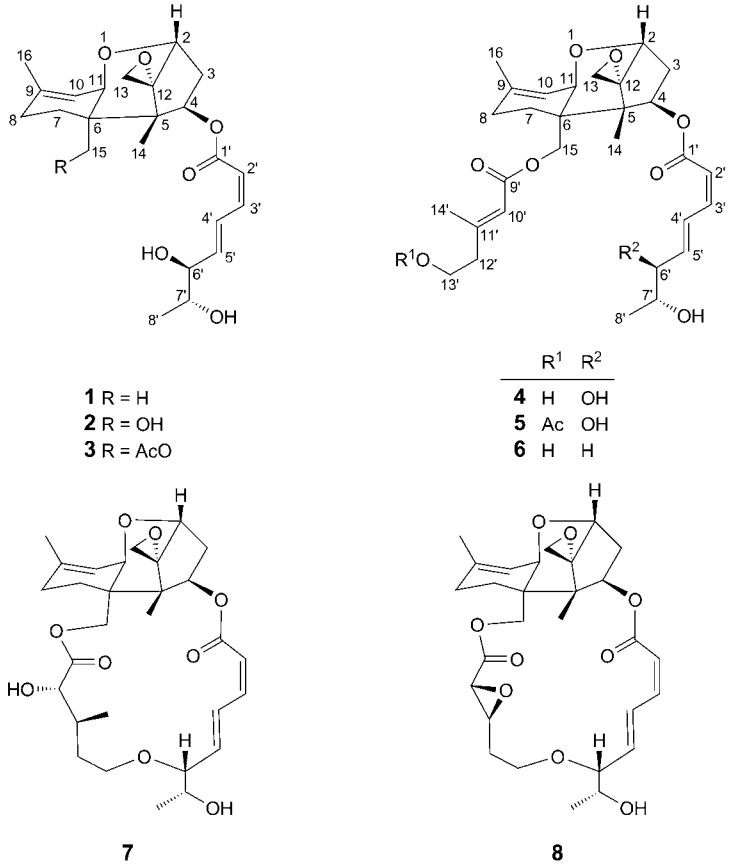

Fig. 1. *Structures of compounds* **1**–**8**

Results and Discussion. – The liquid from 40 l of fermentation broth of *M. roridum* QB-1 was extracted with AcOEt, and then, the crude extract was fractioned by flash silica gel and *Sephadex LH-20* chromatography and semipreparative HPLC to afford three new simple trichothecenes, 15-acetyltrichoverrol B (**3**), 13′-acetyltrichoverrin B (**5**), and 6′-dehydroxytrichoverrin B (**6**), together with five known trichothecenes, trichodermadienediol B (**1**), trichoverrol B (**2**), trichoverrin B (**4**), and roridins A (**7**) and D (**8**).

15-Acetyltrichoverrol B (**3**) was assigned the molecular formula $C_{25}H_{34}O_8$, as determined by HR-ESI-MS (*m*/*z* 485.2140 $[M+Na]^+$). The molecular formula differed from that of trichoverrol B (**2**) in having a relative molecular mass 42 amu more, indicating the possible presence of an AcO group. Further in the comparison of ^{1}H- and ^{13}C-NMR spectroscopic data (*Table 1*), an AcO group signal (δ(H) 2.09; δ(C) 21.2 and 170.8) might appear in **3**, reinforcing the above speculation. Additionally, significant HMBCs between H–C(15)/15-AcO revealed that the AcO group was connected to C(15) in **3** (*Fig. 2*). The absolute configuration of the sesquiterpenol

Table 1. *^1H-* (400 MHz) *and ^{13}C-NMR* (100 MHz) *Data for Compounds* **1**–**3** *in $CDCl_3$* (δ in ppm, J in Hz)

Position	**3**		**5**		**6**	
	δ(H)	δ(C)	δ(H)	δ(C)	δ(H)	δ(C)
2	3.87 (*d*, *J* = 5.2)	79.0	3.87 (*d*, *J* = 5.2)	79.0	3.85 (*d*, *J* = 5.2)	79.1
3	2.00–2.06 (*m*), 2.59 (*dd*, *J* = 15.6, 8.0)	36.9	2.00–2.06 (*m*), 2.59 (*dd*, *J* = 15.6, 8.0)	37.0	2.00–2.06 (*m*), 2.58 (*dd*, *J* = 15.6, 8.0)	36.9
4	5.88 (*dd*, *J* = 7.6, 3.2)	75.0	5.91 (*dd*, *J* = 7.6, 3.2)	75.1	6.08 (*dd*, *J* = 7.6, 3.2)	74.9
5		49.0		48.9		48.6
6		43.1		43.2		43.0
7	1.69–1.73 (*m*), 2.00–2.04 (*m*)	21.1	1.70–1.74 (*m*), 2.00–2.03 (*m*)	21.4	1.60–1.66 (*m*), 2.06–2.10 (*m*)	21.7
8	1.98–2.02 (*m*)	27.9	1.98–2.02 (*m*)	27.9	1.98–2.02 (*m*)	27.9
9		141.0		140.9		140.8
10	5.45 (*d*, *J* = 5.2)	118.2	5.45 (*d*, *J* = 5.2)	118.3	5.48 (*d*, *J* = 5.2)	118.5
11	3.79 (*d*, *J* = 5.2)	66.7	3.81 (*d*, *J* = 5.2)	66.8	3.94 (*d*, *J* = 5.6)	66.7
12		65.9		65.9		65.6
13	2.88 (*d*, *J* = 4.0), 3.19 (*d*, *J* = 4.0)	48.2	2.88 (*d*, *J* = 4.0), 3.19 (*d*, *J* = 4.0)	48.2	2.84 (*d*, *J* = 4.0), 3.16 (*d*, *J* = 4.0)	48.2
14	0.82 (*s*)	7.1	0.83 (*s*)	7.0	0.82 (*s*)	6.8
15	4.07 (*d*, *J* = 12.0), 4.17 (*d*, *J* = 12.0)	63.7	4.05 (*d*, *J* = 12.0), 4.24 (*d*, *J* = 12.0)	63.0	4.12 (*d*, *J* = 12.0), 4.16 (*d*, *J* = 12.0)	63.3
16	1.72 (*s*)	23.2	1.72 (*s*)	23.2	1.72 (*s*)	23.2
1′		165.8		165.7		166.2
2′	5.69 (*d*, *J* = 11.2)	118.3	5.69 (*d*, *J* = 11.2)	118.3	5.62 (*d*, *J* = 11.2)	116.4
3′	6.63 (*dd*, *J* = 11.2, 11.2)	143.5	6.62 (*dd*, *J* = 11.2, 11.2)	143.5	6.58 (*dd*, *J* = 11.2, 11.2)	145.1
4′	7.58 (*dd*, *J* = 15.6, 11.2)	128.0	7.59 (*dd*, *J* = 15.6, 11.2)	128.1	7.46 (*dd*, *J* = 15.6, 11.2)	129.6
5′	6.13 (*dd*, *J* = 15.6, 5.6)	140.9	6.13 (*dd*, *J* = 15.6, 5.6)	140.9	6.10 (*dd*, *J* = 15.6, 7.2)	140.8
6′	4.26–4.30 (*m*)	75.5	4.24–4.28 (*m*)	75.5	2.31–2.42 (*m*)	42.3
7′	3.93 (*dq*, *J* = 6.8, 3.6)	70.2	3.93 (*dq*, *J* = 6.8, 3.6)	70.2	3.94–4.00 (*m*)	66.9
8′	1.16 (*d*, *J* = 6.8)	17.8	1.16 (*d*, *J* = 6.8)	17.8	1.22 (*d*, *J* = 6.4)	23.2
9′				165.8		166.0
10′			5.74 (*s*)	117.2	5.85 (*s*)	117.0
11′				156.2		157.1
12′			2.47 (*t*, *J* = 6.4)	39.5	2.39–2.45 (*m*)	43.7
13′			4.21–4.25 (*m*)	61.9	3.77–3.87 (*m*)	59.7
14′			2.20 (*s*)	18.9	2.20 (*s*)	19.1
15-AcO		170.8				
	2.09 (*s*)	21.2				
13′-AcO				171.3		
			2.04 (*s*)	20.9		

substructure of **3** was elucidated as (2*R*,4*R*,5*S*,6*R*,11*R*,12*S*) by the corresponding NMR data, which were nearly identical to those of trichoverrol B (**2**). This assumption was also supported by the NOESY correlations of H–C(4)/H–C(11)/H–C(15) and H–C(2)/H–C(13)/Me(14) (*Fig. 3*). Finally, compound **3** was shown to have most likely (6′*S*,7′*R*)-configuration by a *doublet* of *quartet*s for the signal of H–C(7′) appearing as an eight-line signal ($J(6',7') = 3.6$ Hz, $J(7',8') = 6.8$ Hz) and the same corresponding ^{13}C-NMR data at C(6′) and C(8′) as the B-series trichoverroids, which resonated *ca.*

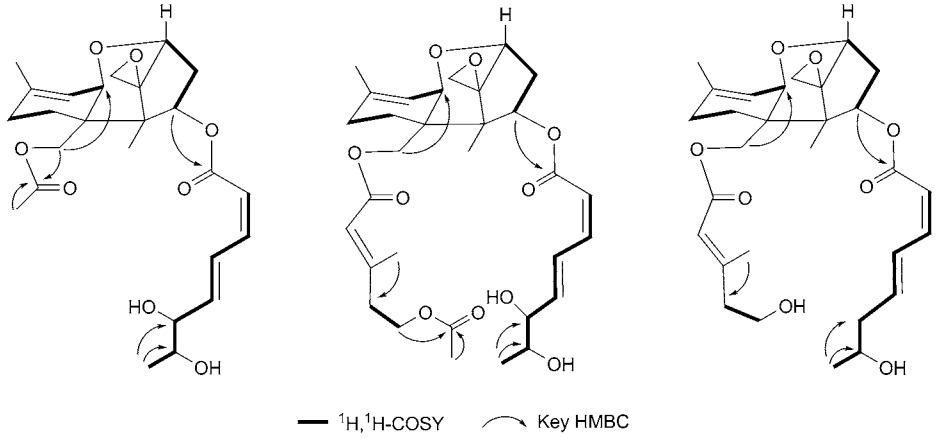

Fig. 2. *Key ^{1}H,^{1}H-COSY and HMBC of* **3**, **5**, *and* **6** (COSY correlations are indicated by bold lines)

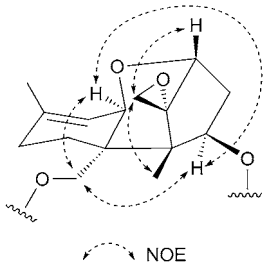

Fig. 3. *Selected NOESY correlations of trichothecene ring of* **3**

1 ppm upfield relative to the resonances for these C-atoms observed for A-series. Additionally, the configuration of C(6′) in roridin A (**7**) is (*R*), and the ring closure from the trichoverrins to the macrocycles has occurred with inversion of configuration at C(6′) [11], indicating that the the configuration of C(6′) in **3** was (*S*). Therefore, the absolute configuration of 15-acetyltrichoverrol B (**3**) was identical to **1**, **2**, and **4**, which was determined as (2*R*,4*R*,5*S*,6*R*,11*R*,12*S*,6′*S*,7′*R*).

13′-Acetyltrichoverrin B (**5**) displayed an $[M+Na]^+$ ion at *m*/*z* 597.2683 in the HR-ESI-MS, consistent with a molecular formula of $C_{31}H_{42}O_{10}$. The molecular formula had a relative molecular mass 42 amu more than that of trichoverrin B (**4**). The ^{1}H- and ^{13}C-NMR data of **5** shown in *Table 1* revealed the similarity to trichoverrin B (**4**), except for the presence of one Ac group signal (δ(H) 2.04; δ(C) 20.9 and 171.3). Therefore, compound **5** was deduced as being a monoacetate of trichoverrin B (**4**), which was supported by distinct HMBCs between H–C(13′)/13′-AcO (*Fig. 2*), which proved the AcO group to be attached to C(13′). The configuration of **5** was assumed to be identical to that of **3** due to their common biosynthetic origin, which also was supported by the NOESY data and the same corresponding 1D-NMR data at sesquiterpene ring, C(6′) and C(8′). Therefore, the absolute structure of 13′-acetyltrichoverrin B (**5**) was also assigned as (2*R*,4*R*,5*S*,6*R*,11*R*,12*S*,6′*S*,7′*R*).

6′-Dehydroxytrichoverrin B (**6**), isolated as oil, was determined to be $C_{29}H_{40}O_8$ on the basis of HR-ESI-MS (*m/z* 539.2620 $[M+Na]^+$). The molecular formula had a relative molecular mass 16 amu less than that of trichoverrin B (**4**), indicating the possible absence of a OH group. Comparing the ^{1}H-NMR spectra of **6** (*Table 1*) and **4**, the OH group which resonated at δ(H) 4.27 in **4** had disappeared in **6**. Further, the ^{1}H,^{1}H-COSY correlations of H–C(6′)/H–C(7′)/H–C(8′) (*Fig. 2*) and the CH_2 signal (δ(H) 2.31 – 2.42; δ(C) 42.3) at C(6′) in **6** reinforced the above supposition, which also was supported by significant HMBCs between H–C(8′)/C(6′), C(7′) (*Fig. 2*). From the biosynthetic standpoint, the absolute configuration of 6′-dehydroxytrichoverrin B (**6**) was assigned as (2*R*,4*R*,5*S*,6*R*,11*R*,12*S*,7′*R*).

All the compounds were evaluated for antifungal (*Phytophthora capsici*, *Fusarium moniliforme*, *Alternaria solani*, *Fusarium graminearum*, *Fusarium coeruleum*, and *Piricularia Oryzae* CGMCC:3.3283) activities. Only compounds **7** and **8** showed significant antifungal activity against *F. graminearum*, while the others were inactive (*MIC* > 10 μg/ml; *Table 2*).

Table 2. *The MIC Values of Compounds* **3** *and* **5**–**8** [μg/ml]

Target fungal	Antifungal activity against *F. graminearum* (*MIC* [μg/ml])
3	NA[a])
5	NA
6	NA
7	5
8	10
Amphotericin B[b])	1.25[c])

[a]) NA = not active (*MIC* > 10 μg/ml). [b]) Positive control. [c]) The value tested, similar to 1.82 in [13].

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Experimental Part

General. The semi-prep. HPLC was accomplished over a *Hypersil ODS* column (5 μm, 250 mm × 10 mm, *Thermo Fisher Scientific*, USA) on a *Hitachi* HPLC system consisted of a *L-7110* pump (*Hitachi*) with a *L-7420 UV-VIS* Detector (*Hitachi*). Silica gel (SiO_2; 200 – 300 mesh) for column chromatography (CC) and silica gel GF_{254} for TLC were produced by *Qingdao Marine Chemical Company* (P. R. China). *Sephadex LH-20* was purchased from *Pharmacia Biotech* (Sweden). All chemicals used in the study were of analytical grade. Optical rotations: *Rudolph Autopol III* automatic polarimeter. IR Spectra: *Nexus 870 FT-IR* spectrometer. UV Spectra: *Hitachi U-3000* spectrophotometer in MeOH. NMR Spectra: *Bruker DRX 400* spectrometer equipped with a 5 mm inverse probe and operating at 400 MHz for ^{1}H-NMR and 100 MHz for ^{13}C-NMR. $CDCl_3$ for NMR measurements was purchased from *Sigma–Aldrich Chemicals.* HR-ESI-MS Spectra: *Agilent 6210 TOF LC/MS* equipped with an electrospray ionization (ESI) probe operating in positive- or negative-ion mode with direct infusion.

Biological Material. The *M. roridum* QB-1 was separated from *Raphanus sativus* L. collected in October 2013, from the suburb of Jurong in Jiangsu Province (P. R. China). The *M. roridum* stain was identified by analyzing the morphological characters on PDA medium, comparing with two *M. roridum* strains, IFB-E009 and IFB-E012 which had been identified by 18SrDNA sequence with those of standard records [10]. The conidia of the *M. roridum* QB-1 were single-celled, olive-brown, and cylindrical with

both ends rounded (5.0–7.2 × 2.0 μm), similar to the *M. roridum* strains, IFB-E009 and IFB-E012. The live culture of the *M. roridum* strain was kept at the Institute of Functional Biomolecules, Nanjing University (P. R. China).

Fermentation. The *M. roridum* was cultured in liquid medium. The fresh mycelium grown on PDA medium at 28° for 4 d was inoculated into flasks (1000 ml) containing CzapekAs medium (400 ml from a mixture of sucrose (30 g), $NaNO_3$ (3 g), K_2HPO_4 (1 g), yeast extract (1 g), KCl (0.5 g), $MgSO_4 \cdot 7\,H_2O$ (0.5 g), $FeSO_4$ (0.01 g), and H_2O (1000 ml)). After 4 d of the incubation at 28° on a rotary shaker at 120 rpm, a portion of the liquid culture (2000 ml) was transferred as the seed into each of a total of 100 flasks (1000 ml) containing CzapekAs medium (400 ml). Fermentation was carried out on a rotary shaker (120 rpm) at 28° for 12 d with humidity in the range 60–70%.

Extraction and Isolation. The obtained biomasses of the fermented culture was extracted exhaustively with AcOEt at r.t., and the org. solvent was evaporated to dryness under reduced pressure to afford a brown crude extract (7.0 g), which gave three fractions (*Fr. 1*, 3.1 g; *Fr. 2*, 2.0 g; *Fr. 3*, 1.9 g) after CC (6 × 60 cm) over SiO_2 (70 g, 200–300 mesh) eluted with a gradient of CH_2Cl_2/MeOH (*v*/*v* 100:0, 100:1, 100:2, 100:4, 100:8, 0:100, each 1400 ml) based on TLC monitorings. *Fr. 1* was further purified by using *Sephadex LH-20* with CH_2Cl_2/MeOH (1:1) and HPLC (68% MeCN in H_2O) to give **7** (20.5 mg, t_R = 7.2 min) and **8** (10.3 mg, t_R = 8.1min). *Fr. 2* was further purified by using *Sephadex LH-20* with CH_2Cl_2/MeOH (1:1) and HPLC (55% MeCN in H_2O) to give **2** (10.1 mg, t_R = 7.2 min) and **4** (8.3 mg, t_R = 12.5 min). Similarly, purification of subfraction *Fr. 3* with *Sephadex LH-20* with CH_2Cl_2/MeOH (1:1) and HPLC (50% MeCN in H_2O) afforded **3** (15.5 mg, t_R = 11.2 min), **1** (5.5 mg, t_R = 13.5 min), **6** (6.2 mg, t_R = 15.2 min), and **5** (5.3 mg, t_R = 17.1 min).

15-Acetyltrichoverrol B (= *(4β)-15-(Acetyloxy)-12,13-epoxytrichothec-9-en-4-yl (2Z,4E,6S,7R)-6,7-Dihydroxyocta-2,4-dienoate*; **3**). Oil. $[\alpha]_D^{28} = +1.4$ (*c* = 0.47, MeOH). UV (MeOH): 206 (4.04), 261 (3.80). IR (KBr): 3433, 2970, 2936, 1740, 1713, 1640, 1383, 1242, 1179, 1079, 966. ^{1}H- and ^{13}C-NMR: see *Table 1*. HR-ESI-MS: 485.2140 ($[M + Na]^+$, $C_{25}H_{34}NaO_8^+$; calc. 485.2151).

13′-Acetyltrichoverrin B (= *(4β)-15-{[(2E)-5-(Acetyloxy)-3-methylpent-2-enoyl]oxy}-12,13-epoxytrichothec-9-en-4-yl (2Z,4E,6S,7R)-6,7-Dihydroxyocta-2,4-dienoate*; **5**). Oil. $[\alpha]_D^{28} = +6.8$ (*c* = 0.13, MeOH). UV (MeOH): 206 (4.12). IR (KBr): 3432, 2968, 2932, 1715, 1647, 1384, 1228, 1148, 1079, 967. ^{1}H- and ^{13}C-NMR: see *Table 1*. HR-ESI-MS: 597.2683 ($[M + Na]^+$; $C_{31}H_{42}NaO_{10}^+$, 597.2676).

6′-Dehydroxytrichoverrin B (= *(4β)-15-{[(2E)-5-Hydroxy-3-methylpent-2-enoyl]oxy}-12,13-epoxytrichothec-9-en-4-yl (2Z,4E,7R)-7-Hydroxyocta-2,4-dienoate*; **6**). Oil. $[\alpha]_D^{28} = -10.4$ (*c* = 0.17, MeOH). UV (MeOH): 214 (4.15), 262 (4.03). IR(KBr): 3422, 2966, 2931, 1713, 1641, 1383, 1280, 1223, 1145, 1078, 966. ^{1}H- and ^{13}C-NMR: see *Table 1*. HR-ESI-MS: 539.2620 ($[M + Na]^+$, $C_{29}H_{40}NaO_8^+$; calc. 539.2621).

Antifungal Activity Assay. The antifungal activities were evaluated against six fungal strains (*P. capsici*, *F. moniliforme*, *A. solani*, *F. graminearum*, *F. coeruleum*, and *P. Oryzae* CGMCC:3.3283), which were obtained from the College of Plant Protection, Nanjing Agricultural University. The antifungal assay was performed as described previously [14]. The broth dilution method using 96-well microtiter plates was applied, and the final concentration of test compounds ranged from 10 to 5 μg/ml. Amphotericin B was used as positive control. The *MIC* was determined for three times as the lowest concertration at which no growth was observed.

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