Cytotoxic Sorbicillinoids and Bisorbicillinoids from a Marine-Derived Fungus *Trichoderma* sp.

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Four sorbicillinoids (1–4) and seven bisorbicillinoids (5–11), including two new compounds, 6-demethylsorbicillin (1) and 10,11-dihydrobisvertinolone (6), were isolated from a marine-derived fungus *Trichoderma* sp. Their cytotoxic activities against HL-60 cell line were evaluated by Sulforhodamine B (SRB) assay method and flow cytometric analysis.

Key words *Trichoderma* sp.; sorbicillinoid; bisorbicillinoid

The “sorbicillinoids” and “bisorbicillinoids”¹⁾ are defined to designate a group of more than 30²⁾ monomeric and dimeric sorbicillin^{3,4)}-related natural products, which are produced by a few fungal genera (*Trichoderma*, *Verticillium*, and *Penicillium*).⁵⁾ The dimers form two groups: the Diels–Alder products such as bisorbicillinol (9) and the Michael-type adducts which are at least tricyclic as in bisvertinolone (5).²⁾ All monomers and dimers exhibit a pronounced radical-scavenging activity,^{6,7)} and some bisorbicillinoids show promising biological properties such as cytotoxicity,^{8,9)} inhibition of the biosynthesis of β -1,6-glucan¹⁰⁾ and the production of tumor necrosis factor α (TNF- α)¹¹⁾ induced by lipopolysaccharide. Isobisvertinol, a new stereoisomer of bisvertinol, shows unique activity to inhibit lipid droplet accumulation in macrophages.¹²⁾

In our search for antitumor compounds from marine-derived microorganisms, a fungus, authenticated as *Trichoderma* sp., was obtained from the marine sediment collected in Fujian province of China. The isolation work of the active compounds on this fungus led to the finding of two new compounds (1, 6), along with nine known compounds [sohirnones A (2), sorbicillin (3), 2',3'-dihydrosorbicillin (4), bisvertinolone (5), trichodimerol (7), dihydrotrichodimerol (8), bisorbicillinol (9), bisvertinoquinol (10), bisorbibutenolide (11a)].^{2,8,10,13–15)} The known compounds were identified by comparisons with previously reported physical and spectral data. In this paper, we describe the isolation, structure elucidation of two new compounds, and cytotoxic activities against HL-60 cell line of all compounds.

Compound 1 was obtained as a yellow powder. Its molecular formula $C_{13}H_{14}O_3$ was determined by HR-electron spray ionization (ESI)-MS (m/z 219.1017 $[M+H]^+$, Calcd 219.1021), indicating seven degrees of unsaturation. Comparison of the 1H - and ^{13}C -NMR data (Table 1) with those of the known compounds sohirnones A (2),²⁾ sorbicillin (3),¹³⁾ and 2',3'-dihydrosorbicillin (4)²⁾ indicated that they had the same skeleton. Further comparison of the 1D NMR spectra of 1 with those of 3 suggested that 1 possessed a sorbyl group (δ_H 7.46, H-9; 6.35, H-10; 6.30, H-11; 1.91, H-12; δ_C 192.9, C-7; 122.3, C-8; 145.4, C-9; 131.1, C-10; 142.0, C-11; 19.5, C-12) as 3, but one of the aromatic methyl groups of 3 was missing in 1. An additional aromatic proton signal appeared at δ 6.36 and the very small coupling between the two aromatic protons pointed to their *para* orientation as in

2. The structure of 1 was finally confirmed, named 6-demethylsorbicillin. The *trans* configuration of the double bond was shown by the same pattern of the proton signals and nearly identical 1H and ^{13}C shifts for the side chain as in 3.

Compound 6 was obtained as a yellow powder and it was analyzed by HR-ESI-MS (m/z 515.2265 $[M+H]^+$, Calcd 515.2281) for the molecular formula $C_{28}H_{34}O_9$. Its IR spectrum exhibited strong absorptions at 3420 and 1670 cm^{-1} , indicative of hydroxy and conjugated carbonyl groups. Analysis of the ^{13}C -NMR data for 6 revealed 13 quaternary carbons, 7 methines, 2 methylenes and 6 methyls. The 1H - and ^{13}C -NMR data (Table 1) were very similar to those of bisvertinolone (5)¹⁰⁾ and it indicated that they had the same constitution except that one double bond on one of the two side chains in 5 was hydrogenated in 6. The chemical shifts of C-15—C-20 (δ 186.2, 122.4, 149.0, 131.9, 144.8, 19.2) were consistent with those in 5, indicating that the hydrogenation occurred on the other side chain. The doublet peak shapes of CH_3 -14 in 1H -NMR spectrum indicated that the C-12/C-13 double bond should not be hydrogenated. Further comparison of the ^{13}C -NMR data of C-9—C-14 (δ 181.0, 33.5, 30.3, 129.9, 127.1, 18.5) with those of the 10,11,16,17-hydrogenated analog tetrahydrobisvertinolone⁹⁾ could definitely help to determine that the C-10/C-11 double bond was hydrogenated. So the structure of 6 was established as the 10,11-hydrogenated analog of 5, named 10,11-dihydrobisvertinolone. The biosynthesis and the absolute configuration of bisvertinolone (5) produced by *Trichoderma* sp. have been well confirmed by a biosynthetic method using oxosorbicillinol and sorbicillinol as the direct precursors.¹⁶⁾ The absolute configurations of dihydrobisvertinolone and tetrahydrobisvertinolone were consistent with that of bisvertinolone (5) by comparison of their nuclear Overhauser effect spectroscopy (NOESY) and circular dichroism (CD) spectra.⁹⁾ So the absolute configuration of 6 was determined to be the same as 5 on biogenetic ground and by their similar 1H and ^{13}C shifts and specific rotations (-340° for 6, -495° for 5, lit.¹⁰⁾).

The cytotoxic effects of compounds 1–11 were preliminarily evaluated against HL-60 cell line using VP16 as positive control. As shown in Table 2, compounds 5 and 7 displayed relatively more potent cytotoxic activities compared with the other compounds. Notably, both monomers and

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Table 1. ^1H - (600 MHz) and ^{13}C - (150 MHz) NMR Data for Compounds **1** and **6** in CDCl_3

No.	1		6	
	δ_{C}	δ_{H}	δ_{C}	δ_{H}
1	165.2		99.6	
1a			55.5	3.67 (1H, s)
2	114.8		191.1	
3	132.5	7.55 (1H, s)	110.7	
4	116.3		164.2	
4a			80.5	
5a			104.6	
5	161.5		79.6	
6	103.8	6.36 (1H, s)	197.1	
7	192.9		107.7	
8	122.3	6.93 (1H, d, 14.6)	200.5	
8a			60.1	
9	145.4	7.46 (1H, dd, 14.6, 10.5)	181.0	
10	131.1	6.35 (1H, m)	33.5	2.49 (1H, m), 2.64 (1H, m)
11	142.0	6.30 (1H, m)	30.3	2.40 (1H, m)
12	19.5	1.91 (3H, d, 6.4)	129.9	5.50 (1H, m)
13			127.1	5.50 (1H, m)
14			18.5	1.63 (3H, d, 5.5)
15			186.2	
16			122.4	7.40 (1H, d, 14.8)
17			149.0	7.59 (1H, dd, 14.8, 9.9)
18			131.9	6.35 (1H, m)
19			144.8	6.35 (1H, m)
20			19.2	1.92 (3H, d, 4.9)
CH_3 -3			7.5	1.51 (3H, s)
CH_3 -4	15.8	2.20 (3H, s)		
CH_3 -4a			25.9	1.49 (3H, s)
CH_3 -5			23.6	1.40 (3H, s)
CH_3 -8a			19.8	1.44 (3H, s)
OH-1		13.27 (1H, s)		
OH-9				17.75 (1H, s)
OH-15				16.66 (1H, s)

Fig. 1. Structures of Compounds **1**–**11**

dimers with full-unsaturated side chains all showed higher cytotoxicity than the corresponding dihydro analogs. Comparison of the percentage of cells with the hypodiploid DNA content determined by flow cytometry (Table 2, Fig. 2) also supported the deduction, suggesting that the conjugated double bonds in side chain might favor the cytotoxic activity of both sorbicillinoids and bisorbicillinoids. Exceptionally, the Diels–Alder products **9**–**11** all exhibited scarcely any activ-

ity at $50\ \mu\text{M}$, probably due to the noneffective molecular architecture. Detailed studies on their antitumor activities and related mechanisms of action are being undertaken.

Experimental

General Experimental Procedures Specific rotations were obtained on a JASCO P-1020 digital polarimeter. UV spectra were recorded on Beckman DU® 640 spectrophotometer. IR spectra were taken on a NICOLET NEXUS 470 spectrophotometer in KBr discs. ^1H -, ^{13}C -NMR and distortionless en-

Fig. 2. Determination of Sub-G₁ Fractions by Flow Cytometric Analysis

Table 2. Cytotoxic Activity of **1**–**11** against HL-60 Cell Line

Compd.	HL-60 (IC ₅₀ , μ M) ^{a)}	Sub-G ₁ (%) ^{b)}
1	23.9	66.4
2	>50	5.4
3	12.7	79.9
4	>50	4.5
5	5.3	89.6
6	49.0	22.6
7	7.8	89.3
8	36.4	55.7
9	>50	7.3
10	>50	4.0
11	>50	8.0
Positive control (VP 16)	2.1	
Negative control (MeOH)		2.3

a) IC₅₀ is defined as the concentration that results in a 50% decrease of viable cell numbers. Data represent mean values of three independent experiments and were determined by the SRB method. b) Data were obtained from histograms. The percentage of sub-G₁ fractions was analyzed using the CellQuest computer software.

hancement by polarization transfer (DEPT) spectra and 2D-NMR were recorded on a JEOL JNM-ECP 600 spectrometer using TMS as internal standard and chemical shifts were recorded as δ values. ESI-MS was measured on a Q-TOF ULTIMA GLOBAL GAA076 LC mass spectrometer. Semipreparative HPLC was performed using an ODS column [YMC-pak ODS-A, 10 $\times$ 250 mm, 5 μ m, 4 ml/min].

Fungal Material The fungus, strain f-13, was obtained from the marine sediment collected in Fujian province of China. It was identified as *Trichoderma* sp. on the basis of its ribosomal internal transcribed spacers and the 5.8S ribosomal RNA gene (ITS1-5.8S-ITS2). Working stocks were prepared on Potato Dextrose agar slants stored at 4 °C in our laboratory.

Fermentation, Extraction, and Isolation Spores were directly inoculated into 500 ml Erlenmeyer flasks containing 200 ml fermentation media (mannitol 20 g, maltose 20 g, glucose 10 g, monosodium glutamate 10 g, KH₂PO₄ 0.5 g, MgSO₄·7H₂O 0.3 g, yeast extract 3 g and corn steep liquor 1 g, dissolved in 1 l seawater, pH 6.5). The flasks were incubated on a rotary shaker at 165 rev./min at 28 °C. After 12 d of cultivation, 80 l of whole broth was filtered through cheesecloth to separate the broth supernatant and mycelia. The former was extracted with EtOAc while the latter was extracted

with acetone. The acetone extract was evaporated under reduced pressure to afford an aqueous solution, and then extracted with EtOAc. The two EtOAc extracts were combined and concentrated *in vacuo* to give a crude gum (90 g).

The crude gum (90 g) was subjected to silica gel column chromatography (petroleum ether–acetone, v/v, gradient) and the active fractions 2 and 3 eluted with the solvent of petroleum ether–acetone (8 : 2 and 7 : 3) were separately subjected to repeating Sephadex LH-20 column chromatography (chloroform–methanol, 1 : 1). The active subfraction 2-7 was recrystallized to give compounds **7** (450 mg). The other active subfractions 2-2, 3-4-3, and 3-5-7 were further purified respectively by HPLC using a reversed-phase C₁₈ column (70/30 MeOH/H₂O, 80/20 MeOH/3% THF, and 70/30 CH₃OH/0.3% THF, 4.0 ml/min), to give compounds **1** (3.4 mg), **2** (6.5 mg), **3** (33.5 mg) and **4** (5.3 mg); **5** (135.4 mg), **6** (6.5 mg), **8** (25.5 mg) and **10** (15.3 mg); **9** (93.2 mg) and **11** (84.1 mg).

6-Demethylsorbicillin (1): Yellow solid (methanol), IR (KBr) cm⁻¹: 3419, 2916, 1640, 1625, 1558, 1485, 1385, 1362, 1285, 1235, 1180, 1154, 1093, 1072, UV $\lambda_{\max}$ (MeOH) nm (log ϵ): 307 (4.10), 207 (4.08), ¹H-NMR (CDCl₃, 600 MHz) and ¹³C-NMR (CDCl₃, 150 MHz), see Table 1, HR-ESI-MS *m/z*: 219.1017 [M+H]⁺ (Calcd for C₁₃H₁₅O₃: 219.1021).

10,11-Dihydrobisvertinolone (6): Yellow solid (methanol), [α]_D²⁰ –340° (c=0.32, MeOH), IR (KBr) cm⁻¹: 3420, 2935, 1670, 1601, 1547, 1448, 1379, 1345, 1212, 1100, 1154, 1026, UV $\lambda_{\max}$ (MeOH) nm (log ϵ): 358 (4.17), 307 (sh, 4.07), 229 (4.06), 207 (4.04), ¹H-NMR (CDCl₃, 600 MHz) and ¹³C-NMR (CDCl₃, 150 MHz), see Table 1, HR-ESI-MS *m/z*: 515.2265 [M+H]⁺ (Calcd for C₂₈H₃₅O₉: 515.2281).

In Vitro Cytotoxicity Assays In the Sulforhodamine B (SRB) assay,¹⁷⁾ cell suspensions (200 μ l) were plated in 96-well plates at a density of 2 $\times$ 10⁵ cells/ml. Then the test compound solutions (2 μ l in MeOH) at different concentrations were added to each well and further incubated for 24 h. Following drug exposure, the cells were fixed with 12% trichloroacetic acid and the cell layer was stained with 0.4% SRB. The absorbance of SRB solution was measured at 515 nm. Dose–response curves were generated and the IC₅₀ values were calculated from the linear portion of log dose–response curves.

In the flow cytometric analysis,¹⁸⁾ cells were plated in 24-well cell culture plates (2 $\times$ 10⁵ cells/well), in triplicate, and then treated with MeOH (Control) or the compounds (**1**–**11**) for 24 h at the concentration of 50 μ M. Cells were harvested, washed twice with PBS, and the cellular DNA stained with 200 μ l propidium iodide (PI, 50 μ g/ml, RNase 1 μ g/ml, Triton X-100 0.1%). After incubation at 4 °C for 20 min, the cells were analyzed using flow cytometry (Becton-Dickinson, Vantage, San Diego, CA, U.S.A.).

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