Sesquiterpenoids from the Endophytic Fungus *Trichoderma* sp. PR-35 of *Paeonia delavayi*

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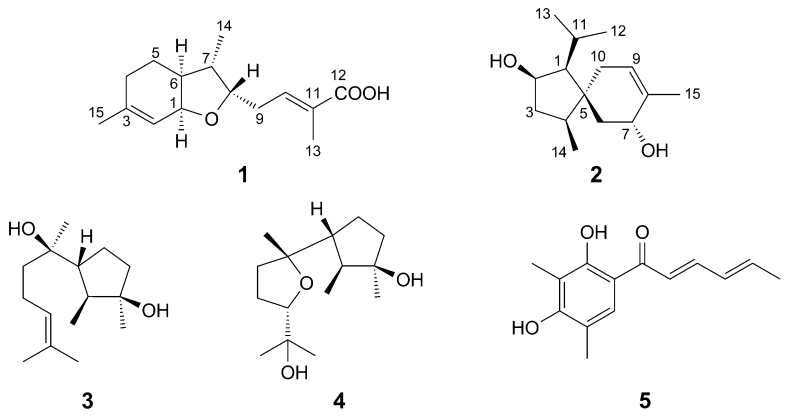
A new bisabolane-type sesquiterpene, trichoderic acid (**1**), and a new acorane-type sesquiterpene, 2β-hydroxytrichoacorenol (**2**), along with three known compounds, cyclonerodiol (**3**), cyclonerodiol oxide (**4**), and sorbicillin (**5**), were isolated from the culture broth of *Trichoderma* sp. PR-35, an endophytic fungus isolated from *Paeonia delavayi*. Their structures were elucidated on the basis of their IR, MS, and 1D- and 2D-NMR analyses. The antibacterial and antifungal activities of **1**–**5** towards various types of bacteria and fungi were tested. Most of the compounds showed moderate or weak antimicrobial activities in an agar-diffusion assay.

Introduction. – Endophytic fungi inhabiting healthy tissues of host plants have been the source of a wide range of novel bioactive natural products [1–3]. *Paeonia delavayi* FRANCH. is an important herb known to be an analgesic, sedative, and anti-inflammatory agent, and also a remedy for cardiovascular, extravasated blood, stagnated blood, and female diseases in traditional oriental medicine [4–6]. Various components, mainly including monoterpenoids, triterpenoids, and flavonoids, have already been isolated from this plant [7]. However, few work has been conducted on the endophytic fungi of the plant.

As part of our ongoing efforts to discover new bioactive secondary metabolites from endophytic fungi of medicinal plants [8][9], we investigated the AcOEt extract of the culture broth of *Trichoderma* sp. PR-35 isolated from the healthy stem of *P. delavayi* and found that it showed potent antimicrobial activity. A follow-up fractionation was, therefore, performed to afford a new bisabolane-type sesquiterpene, trichoderic acid (**1**), and a new acorane-type sesquiterpene, 2β-hydroxytrichoacorenol (**2**), along with three known compounds, cyclonerodiol (**3**), cyclonerodiol oxide (**4**), and sorbicillin (**5**). Here, we report the isolation and structure elucidation of the new compounds as well as their antimicrobial activities towards eight pathogenic microbes. We describe, for the first time, the isolation of secondary metabolites from endophytic fungi of the plant *P. delavayi*.

Results and Discussion. – 1. *Structure Elucidation.* Trichoderic acid (**1**) was isolated as a colorless oil. The HR-ESI-MS showed an $[M-H]^-$ ion peak at m/z 249.1502 (calc. 249.1491), in accord with the molecular formula $C_{15}H_{22}O_3$, implying five degrees of unsaturation. The IR spectrum displayed absorption bands for ester C=O (1688 cm^{-1})



and C=C (1643 cm^{-1}) groups. The ^{1}H-NMR spectrum (*Table 1*) displayed signals of two olefinic H-atoms (δ(H) 7.32 (*t*, *J* = 7.1), 5.55 (br. *s*)), two low-field H-atoms (δ(H) 4.44 (br. *s*), 4.18 (*dt*, *J* = 8.3, 5.6)), and three Me groups (δ(H) 2.06 (*s*), 1.59 (*s*), and 0.91 (*d*, *J* = 7.1)). Signals of one COOH group (δ(C) 171.3) and four olefinic C-atoms (δ(C) 140.2 (*d*), 138.3 (*s*), 130.9 (*s*), and 123.7 (*d*)) were observed in the ^{13}C-NMR and DEPT spectrum (*Table 1*), which were responsible for three out of five unsaturation degrees. The remaining two were ascribed to the presence of two rings. One cyclohexene unit could be deduced from the ^{1}H,^{1}H-COSY spectrum, in which H–C(1) (δ(H) 4.44 (br. *s*)) coupled with H–C(2) (δ(H) 5.55 (br. *s*)) and H–C(6) (δ(H) 1.75–1.77 (*m*)), and H–C(5) coupled with H–C(4) and H–C(6). In addition, the HMBC correlations from H–C(1), H–C(4), and H–C(5) to C(3) (δ(C) 138.3) also confirmed the presence of the cyclohexene unit. On the basis of the HMQC and ^{1}H,^{1}H-COSY spectra, a C-atom chain, CH(6)–CH(7)–CH(8)–CH_2(9)–CH(10), from one methine (H–C(6)) to another olefinic methine (H–C(10)) was established (parts of bold lines in the *Fig.*). The C(1) was connected with C(8) *via* an O-atom to form a five-membered ring, which was confirmed by the HMBC correlations from H–C(8) (δ(H) 4.18 (*dt*, *J* = 8.3, 5.6)) to C(1) (δ(C) 74.9), C(6) (δ(C) 44.9), and C(10) (δ(C) 140.2). The attachment of Me(15) to C(3) was indicated by the HMBC correlations from Me(15) (δ(H) 1.59 (*s*)) to C(2) (δ(C) 123.7), C(3) (δ(C) 138.3), and C(4) (δ(C) 28.5). The correlation peak between Me(14) (δ(H) 0.91 (*d*, *J* = 7.1)) and H–C(7) (δ(H) 1.93–1.96 (*m*)) observed in the ^{1}H,^{1}H-COSY spectrum suggested that Me(14) was attached to C(7). The HMBC correlations from Me(13) (δ(H) 2.06 (*s*)) to C(10) (δ(C) 140.2), C(11) (δ(C) 130.9), and C(12) (δ(C) 171.3) confirmed the attachment of Me(13) and the C(12)OOH group

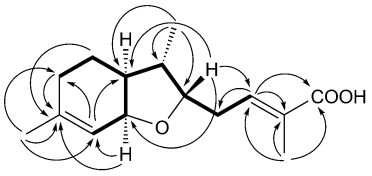


Figure. *^{1}H,^{1}H-COSY* (—) *and key HMBC* (H→C) *correlations of* **1**

Table 1. *^{1}H- and ^{13}C-NMR Data of* **1** and **2**. Recorded at 500 and 125 MHz, respectively, δ in ppm, J in Hz. Atom numbering as indicated in the *Formulae*.

Position	**1**[a])		**2**[b])	
	δ(H)	δ(C)	δ(H)	δ(C)
1	4.44 (br. *s*)	74.9 (*d*)	1.32–1.35 (*m*, overlapped)	68.4 (*d*)
2	5.55 (br. *s*)	123.7 (*d*)	4.20–4.23 (*m*)	74.2 (*d*)
3		138.3 (*s*)	1.67–1.71 (*m*)	41.6 (*t*)
4	1.75–1.77 (*m*, overlapped, H_β), 1.68 (*dd*, J = 17.8, 9.6, H_α)	28.5 (*t*)	1.86–1.89 (*m*, overlapped)	43.8 (*d*)
5	1.56 (*dd*, J = 13.1, 5.6, H_α), 1.38 (*ddd*, J = 13.1, 9.6, 8.8, H_β)	25.1 (*t*)		47.2 (*s*)
6	1.75–1.77 (*m*, overlapped)	44.9 (*d*)	1.79–1.82 (*m*, H_α), 1.32–1.35 (*m*, overlapped, H_β)	34.3 (*t*)
7	1.93–1.96 (*m*)	41.2 (*d*)	4.25 (br. *s*)	69.0 (*d*)
8	4.18 (*dt*, J = 8.3, 5.6)	79.6 (*d*)		135.7 (*s*)
9	2.44–2.50 (*m*, H_a), 2.31–2.36 (*m*, H_b)	32.2 (*t*)	5.48 (br. *s*)	124.9 (*d*)
10	7.32 (*t*, J = 7.1)	140.2 (*d*)	2.17 (br. *d*, J = 10.5, H_α), 1.82–1.85 (*m*, H_β)	36.9 (*t*)
11		130.9 (*s*)	1.86–1.89 (*m*, overlapped)	28.0 (*d*)
12		171.3 (*s*)	1.02 (*d*, J = 6.6)	22.0 (*q*)
13	2.06 (*s*)	13.9 (*q*)	0.98 (*d*, J = 6.6)	25.5 (*q*)
14	0.91 (*d*, J = 7.1)	15.1 (*q*)	0.86 (*d*, J = 6.7)	14.5 (*q*)
15	1.59 (*s*)	24.6 (*q*)	1.76 (*s*)	19.4 (*q*)

[a]) In C_5D_5N. [b]) In $CDCl_3$.

to the olefinic quaternary C(11). The large coupling constants of H–C(5) with H–C(4) ($J(5\beta,4\alpha) = 9.6$) and H–C(5) with H–C(6) ($J(5\beta,6\alpha) = 8.8$) indicated that all of H_α–C(4), H_β–C(5), and H–C(6) are axial. The ROESY correlations observed from H_β–C(5) to H–C(7) and H–C(8) indicated the orientation of H–C(7), H–C(8), and Me(14) to be β, β, and α, respectively. The ROESY correlations from Me(14) to H–C(1) and H–C(6) suggested that H–C(1) and H–C(6) were both α-oriented. Compound **1**, which belongs to the bisabolane sesquiterpene family, was named trichoderic acid.

The bisabolanes are a large group of sesquiterpenes mainly found as constituents of higher plants, and, for many of them, various biological activities have been reported [10]. Few bisabolanes containing the 1,8-epoxy bicyclic structure have previously been reported. Examples include cheimonophyllol E and cheimonophyllal possessing nematicidal and antimicrobial activities, which have been isolated from cultures of the higher fungus *Cheimonophyllum candidissimum* [10][11], and lipistirone isolated from higher fungus *Lepista irina* [12]. Interestingly, this is the first report of a bisabolane-type sesquiterpene from an endophytic fungus.

Compound **2** was isolated as a colorless oil. The molecular formula $C_{15}H_{26}O_2$ was deduced from HR-ESI-MS (*m/z* 239.2000 ($[M+H]^+$; calc. 239.2011)). The IR spectrum showed absorption bands for C=C (1665 cm^{-1}) and OH (3426 cm^{-1}) groups. The ^{1}H-NMR spectrum of **2** (*Table 1*) revealed the presence of an olefinic H-atom (δ(H) 5.48 (br. *s*)), two O-bearing CH groups (δ(H) 4.20–4.23 (*m*) and 4.25 (br. *s*)), a tertiary Me group (δ(H) 1.76 (*s*)), and three secondary Me groups (δ(H) 0.86 (*d*, *J* = 6.7), 0.98 (*d*, *J* = 6.6), and 1.02 (*d*, *J* = 6.6)). The ^{13}C-NMR (DEPT) spectrum of **2** (*Table 1*) exhibited 15 resonances ascribed to four Me, three CH_2, six CH (two oxygenated and one olefinic) groups, and two quaternary C-atoms (one olefinic). These data showed similarities to those of trichoacorenol [13], except for the absence of the CH_2 group with a signal at δ(C) *ca.* 27, and the presence of an additional O-bearing CH group with a signal at δ(C) 74.2. The HMQC spectrum showed that the C-atom at δ(C) 74.2 corresponded to the CH H-atom at δ(H) 4.20–4.23 (*m*). The correlations from the H-atom with the signal at δ(H) 4.20–4.23 (*m*) to H–C(1) (δ(H) 1.32–1.35 (*m*)) and H–C(3) (δ(H) 1.67–1.71 (*m*)) were observed in the ^{1}H,^{1}H-COSY spectrum. The long-range correlations from the signal at δ(H) 4.20–4.23 to C(4) (δ(C) 43.8 (*d*)) and C(11) (δ(C) 28.0 (*d*)) were also observed in the HMBC experiment. Therefore, the CH signal at δ(C) 74.2 was assigned to C(2), substituted by an OH group. The ROESY correlation between H–C(1) and H–C(2) indicated that the OH–C(2) group must be β-oriented. Thus, the structure of **2** was established as 2β-hydroxytrichoacorenol.

The known compounds were identified as cyclonerodiol (**3**) [14], cyclonerodiol oxide (**4**) [15], and sorbicillin (**5**) [16], by comparing their physicochemical properties and NMR spectral data with those reported in the literature. Interestingly, the sesquiterpenes isolated from the endophytic fungus *Trichoderma* sp. PR-35 belong to three structural types, bisabolane (**1**), acorane (**2**), and cyclonerane (**3** and **4**), which reveals the diversity of its terpenic constituents.

2. *Antimicrobial Properties.* Compounds **1**–**5** were tested for their antimicrobial properties against three pathogenic bacteria and five pathogenic fungi by means of the paper-disk diffusion method. The bacteria included *Escherichia coli*, *Staphylococcus albus*, and *Shigella sonnei*, and the fungi tested were *Botrytis cinerea*, *Fusarium avenaceum*, *Fusarium moniliforme*, *Hormodendrum dermatitidis*, and *Pyricularia oryzae*. The results, expressed in terms of minimal inhibitory amount (*MIA*; in μg/disk), are collected in *Table 2*, with penicillin and nystatin as positive control for bacteria and fungi, respectively.

As shown in *Table 2*, all of the test compounds exhibited antibacterial activities against *E. coli* and *S. albus* with *MIA* values in the range of 25–150 μg/disk; compound **1** was the most potent with a value of 25 μg/disk. As a whole, all five compounds showed more potent antibacterial activities than antifungal activities. Compounds **1**, **3**, and **4** exhibited a similar inhibition spectrum on the microbial species with different *MIA* values, even though compound **1** belongs to a different structural type than **3** and **4**. All of the test compounds except for **2** were active against *Shigella sonnei* and *Fusarium avenaceum*. Only compound **2** showed weak antifungal activities against *Botrytis cinerea* and *Pyricularia oryzae* with *MIA* values at 175 and 200 μg/disk, respectively. None of the test compounds showed activity against the *Fusarium moniliforme* at 200 μg/disk. All compounds except for **5** were active against *Hormodendrum dermatitidis* with *MIA* values in the range of 50–100 μg/disk.

Table 2. *Antimicrobial Activities of* **1**–**5** *towards Different Bacterial and Fungal Strains.* All values are expressed in terms of minimal inhibitory amount (*MIA* in μg/disk). For details, see *Exper. Part.*

Organism	**1**	**2**	**3**	**4**	**5**	Penicillin[a])	Nystatin[b])
Escherichia coli	25	50	75	50	150	6.25	–
Staphylococcus albus	25	150	50	50	150	6.25	–
Shigella sonnei	100	n.a.[c])	125	150	200	12.5	–
Botrytis cinerea	n.a.	175	n.a.	n.a.	n.a.	–	25
Fusarium avenaceum	125	n.a.	125	125	175	–	25
Fusarium moniliforme	n.a.	n.a.	n.a.	n.a.	n.a.	–	12.5
Hormodendrum dermatitidis	75	50	50	100	n.a.	–	6.25
Pyricularia oryzae	n.a.	200	n.a.	n.a.	n.a.	–	25

[a]) Positive control for bacteria. [b]) Positive control for fungi. [c]) Not active.

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Experimental Part

General. TLC: Precoated *Si gel G* plates (*Qingdao Marine Chemical Factory*, Qingdao, P. R. China). Column chromatography (CC): *Diaion HP-20* (*Mitsubishi*); silica gel (SiO_2; 200–300 mesh; *Qingdao Marine Chemical Factory*); *Sephadex LH-20* (*Pharmacia*); reversed-phase C_{18} (*RP-18*) SiO_2 (*Merck*). Optical rotations: *Jasco DIP*-370 polarimeter. UV Spectra: *Shimadzu double-beam 210A* spectrophotometer; λ_{max} (log ε) in nm. IR Spectra: *Bio-Rad FTS* spectrometer, with KBr pellets; in cm^{-1}. NMR Spectra: *Bruker DRX-500* spectrometer; δ in ppm rel. to Me_4Si, *J* in Hz. HR-ESI-MS: *Agilent 3250AA LC/MSD TOF* spectrometer; in *m*/*z*.

Isolation and Identification of the Endophytic Fungus. The fungus was isolated from surface-sterilized fresh stems of an apparently healthy *Paeonia delavayi* collected in September 2007 in Songming County, Yunnan Province, P. R. China. The stems were cut into small rods (*ca.* 8 cm in length) and rinsed in running tap water, followed by successive surface sterilization in 75% EtOH and 0.2% $HgCl_2$, resp., for 1 min each. The stem rods were rinsed three times in sterilized dist. H_2O and cleaved aseptically into small segments, which were then deposited on a *Petri* dish containing PDA medium (200 g potato, 20 g glucose, and 15 g agar in 1 l of H_2O, supplemented with 100 mg/l penicillin to suppress the bacterial growth) and cultivated at 28°. The germinating hypha tips observed were transferred to new PDA plates and then subcultured until pure cultures were obtained. The fungal strain *Trichoderma* sp. PR-35 was isolated by repeated subculturing and deposited with the Yunnan Institute of Microbiology, Kunming, P. R. China. The strain was identified according to its morphological characteristics and ITS sequence data. The colonies on PDA grew fast and covered the whole *Petri* dish after 3 d at 28°, white and cottony. Conidia formed within one week in compact tufts in shades of green. Yellow pigment diffused through the agar. Conidiophores consisted of a strongly developed central axis from which arise, toward the tip, solitary phialides, and further from the tip progressively longer, often paired, secondary branches. Branches arise at or near 90° with respect to the main axis. Each branch terminated in one phialide. Phialides were straight or sinuous, and cylindrical. Phialides formed below the terminus were flask-shaped and enlarged in the middle, constricted to the tip and slightly at the base. Conidia aggregated at the tip of phialides, ellipsoidal, green, and smooth-walled, $2.0–2.5 \times 2.5–5.0$ μm in size. These morphological characteristics enabled the identification of the fungal strain PR-35 as *Trichoderma* sp., which was reinforced by the ITS sequence that gave a 98% sequence similarity to those accessible at the BLASTN of *Trichoderma longibrachiatum* (NCBI accession No. FJ461587).

Cultivation. The fresh mycelium grown on PDA medium at 28° for 6 d was inoculated into 60 × 500 ml *Erlenmeyer* flasks each containing 100 ml of PDB medium (200 g potato and 20 g glucose in 1 l of H_2O). After 4 d of the incubation at 28 ± 1° on a rotary shaker at 200 r.p.m., a 10-ml culture liquid was transferred as seed into each of a total of 600 500-ml *Erlenmeyer* flasks containing 120 ml of PDB medium. The following cultivation was kept for 7 d at 28 ± 1° at 200 r.p.m. on a rotary shaker.

Extraction and Isolation. The cultures (72 l) were filtered to remove mycelia. The culture filtrate was absorbed on a *Diaion HP-20* column (15 l). After the column had been washed with H_2O, the absorbed fraction was eluted with MeOH. By removal of the org. solvent in vacuum, the resulting residue (68 g) was submitted to CC (SiO_2 (1 kg), 200–300 mesh; $CHCl_3$/MeOH 0:1 → 1:0) to afford ten fractions, *Frs. A–J. Fr. B* (2.2 g) was purified by repeated CC (1. SiO_2; petroleum ether (PE)/AcOEt 4:1; 2. *Sephadex LH-20*; MeOH) to yield **5** (7 mg). *Fr. C* (1.4 g) was subjected to CC (SiO_2; PE/AcOEt 9:1, 4:1, and 7:3) to provide three subfractions, *Frs. C.1–C.3. Fr. C.2* was further purified by CC (*Sephadex LH-20*, MeOH) to afford **1** (10 mg). *Fr. D* (3.5 g) was subjected to CC (SiO_2; PE/acetone 95:5, 9:1, and 4:1) to afford four subfractions, *Frs. D.1–D.4. Fr. D.2* was further purified by CC (*RP-18*; MeOH/H_2O 2:3, 1:1, 3:2, and 7:3) to yield **2** (9 mg) and **3** (8 mg). *Fr. D.3* was purified by CC (*Sephadex LH-20*; MeOH) to provide **4** (10 mg).

Trichoderic Acid (=*(2*E*)-4-[(2*S*,3*S*,3a*S*,7a*R*)-2,3,3a,4,5,7a-Hexahydro-3,6-dimethylbenzofuran-2-yl]-2-methylbut-2-enoic Acid*; **1**). Colorless oil. $[\alpha]_D^{26} = -28.4$ ($c = 0.33$, $CHCl_3$). UV ($CHCl_3$): 242 (3.59), 224 (2.37). IR (KBr): 2925, 1688, 1643, 1433, 1289, 1084, 959. ^{1}H- and ^{13}C-NMR: see *Table 1*. HR-ESI-MS: 249.1502 ($[M-H]^-$, $C_{15}H_{21}O_3^-$; calc. 249.1491).

2β-Hydroxytrichoacorenol (=*(1*S*,2*R*,4*S*,5*S*,7*R*)-4,8-Dimethyl-1-(1-methylethyl)spiro[4.5]dec-8-ene-2,7-diol*; **2**). Colorless oil. $[\alpha]_D^{26} = -1.73$ ($c = 0.14$, $CHCl_3$). IR (KBr): 3426, 2967, 1665, 1456, 1374, 1058, 889. ^{1}H- and ^{13}C-NMR: see *Table 1*. HR-ESI-MS: 239.2000 ($[M+H]^+$, $C_{15}H_{27}O_2^+$; calc. 239.2011).

Antimicrobial-Activity Assay. Antibacterial and antifungal activities were determined by the paper-disk diffusion method [17]. The sterilized paper disks (6 mm in diameter) were dipped into a soln. of the test compounds in DMSO at a single dose of 200 μg/disk. The dried disks were then placed in a *Petri* plate containing 10 ml of nutrient agar (NA) (or PDA) medium seeded with 10^6 cells (or spores)/ml suspension of tested bacteria (or fungi) under aseptic conditions. The plates were incubated at 37° for bacteria (18 h) and 28° for fungi (72 h), resp. The inhibition zones were analyzed. Then, the active compounds were resubjected to the same assay at ten different concentrations in the range of 6.25–200 μg/disk. A disk containing only DMSO was used as neg. control. The results were expressed in terms of minimal inhibitory amount (*MIA*; in μg/disk), penicillin (*Wuhan Fude Chemical Co., Ltd.*, Wuhan, P. R. China) and nystatin (*Guangdong Taicheng Parmaceutical Co., Ltd.*, Taishan, P. R. China) being used as pos. control for bacteria and fungi, resp. Each test was performed in triplicate.

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