Three New Sesquiterpenoids from *Xylaria* sp. NCY2

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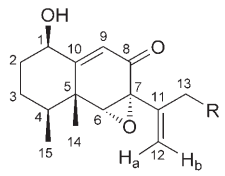
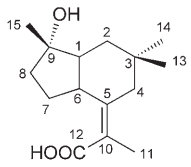
Three new sesquiterpenoids, xylarenones A (**1**) and B (**2**), and xylarenic acid (**3**), were obtained from the endophytic fungal strain *Xylaria* sp. NCY2, which was isolated from *Torreya jackii* CHUN. Their structures were elucidated by spectroscopic analyses, including 1D- and 2D-NMR experiments, and by HR-Q-TOF mass spectrometry. The antitumor and antibacterial properties of the new compounds were evaluated.

Introduction. – Endophytic fungi living in the intracellular spaces of plants are thought to possess different biological properties, and are expected to produce novel and biologically active chemical substances [1–4]. *Torreya jackii* CHUN is an evergreen shrub in the family Taxaceae, and is endemic to southeastern China, being mainly distributed in Zhejiang and Fujian Provinces. In exploring the endophytic fungi of *T. jackii*, we isolated a novel fungal strain with a unique morphology, which was named NCY2, and identified as a *Xylaria* species (Xylariaceae). Herein, we report the isolation, structural determination, and biological properties of three new sesquiterpenes, *i.e.*, xylarenones A (**1**) and B (**2**), and xylarenic acid (**3**), from this fungal material.



1 R = OH
2 R = OAc



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Results and Discussion. – 1. *Structure Elucidation*. The morphological properties of the isolate NCY2 were examined after incubation for 21 d on potato-dextrose agar (PDA) medium at 28°. This organism was identified as a *Xylaria* species, based on its rDNA sequence (ITS1-5.8S-ITS2). The fermentation culture was extracted successively with AcOEt, and the crude extract was purified by repeated column

chromatography (*RP-18*, *Sephadex LH-20*, and silica gel) to afford three new sesquiterpenoids.

Compound **1** was obtained as a colorless oil. The molecular formula was determined as $C_{15}H_{20}O_4$ according to the HR-Q-TOF-MS and NMR data. The IR absorption at 3421 cm^{-1} indicated the presence of OH groups. The ^{13}C-NMR (DEPT) spectrum of **1** (*Table 1*)[1]) exhibited 15 signals: two Me, four CH_2 (one being oxygenated), and four CH groups (two being oxygenated), as well as five quaternary C-atoms, including a C=O function.

Table 1. *^{1}H- and ^{13}C-NMR Data of* **1** *and* **2**. At 500/125 MHz, resp., in $CDCl_3$; δ in ppm, J in Hz.

Position	**1**		**2**	
	δ(C)	δ(H)	δ(C)	δ(H)
1	73.2 (*d*)	4.37 (*s*)	73.6 (*d*)	4.40 (*s*)
2	32.9 (*t*)	2.00 (br. *d*, J = 14.0, H_β), 1.67 (*dt*, J = 14.0, 3.0, H_α)	33.1 (*t*)	1.98–2.01 (*m*), 1.66–1.70 (*m*)
3	24.4 (*t*)	1.89–1.91 (*m*, H_β), 1.45–1.47 (*m*, H_α)	24.4 (*t*)	1.92–1.95 (*m*), 1.45–1.48 (*m*)
4	37.7 (*d*)	1.88–1.90 (*m*)	37.7 (*d*)	1.89–1.91 (*m*)
5	40.1 (*s*)		40.9 (*s*)	
6	69.6 (*d*)	3.36 (*s*)	69.6 (*d*)	3.32 (*s*)
7	62.9 (*s*)		62.2 (*s*)	
8	195.4 (*s*)		193.5 (*s*)	
9	123.0 (*d*)	5.85 (*s*)	123.3 (*d*)	5.87 (*s*)
10	164.2 (*s*)		162.7 (*s*)	
11	142.7 (*s*)		138.5 (*s*)	
12	115.6 (*t*)	5.29 (*s*, H_a), 5.36 (*s*, H_b)	117.2 (*t*)	5.39 (*d*, J = 1.1, H_a), 5.41 (*s*, H_b)
13	63.5 (*t*)	4.33 (*d*, J = 13.5, H_a), 4.18 (*d*, J = 13.5, H_b)	65.1 (*t*)	4.86 (*d*, J = 13.5, H_a), 4.76 (*d*, J = 13.5, H_b)
14	19.1 (*q*)	1.37 (*s*)	19.1 (*q*)	1.39 (*s*)
15	15.8 (*q*)	1.13 (*d*, J = 6.0)	15.8 (*q*)	1.14 (*d*, J = 6.2)
16			170.4 (*s*)	
17			20.9 (*q*)	2.04 (*s*)

The HMBC correlations from the H-atoms of Me(14) to C(4), C(5), C(6), and C(10), from those of Me(15) to C(3), C(4), and C(5), and from H–C(1) to C(2), C(3), C(5), and C(9), along with ^{1}H,^{1}H-COSY correlations, established fragment **A** (*Fig. 1*). Furthermore, the HMBC spectrum showed that H–C(12) was correlated with C(7), C(11), and C(13). In combination with the HMBC correlations from H–C(9) to both C(7) and C(8), this led to the establishment of fragment **B**. Finally, the HMBC correlations from H–C(6) to both C(7) and C(11) required that fragments **A** and **B** were linked through C(6) and C(7). Additionally, the chemical shifts of C(6) (δ(C) 69.6) and C(7) (δ(C) 62.9) were relatively upfield shifted compared to regular oxygenated methines and/or quaternary C-atoms, indicating the presence of an epoxy

[1]) Arbtrary, eremophilane-based atom numbering.

group between C(6) and C(7). This was further supported by HR-MS, which indicated the presence of four rather than five O-atoms in the molecule.

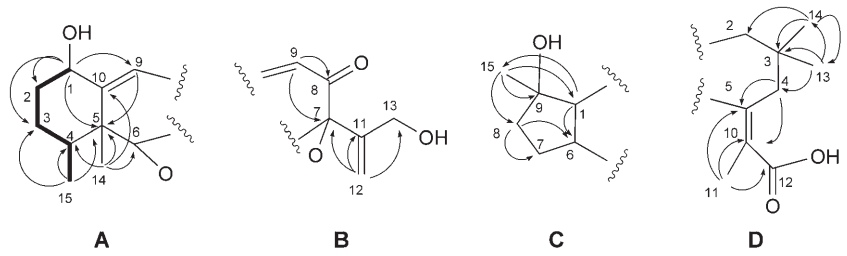

Fig. 1. *Fragments* **A** *and* **B** (for **1** and **2**), *and* **C** *and* **D** (for **3**), *and selected HMBC* (H → C) *and* $^1H,^1H$-*COSY* (▬) *correlations*

The relative configuration of **1** was determined by the analysis of the ROESY spectrum. The presence of ROESY correlations between H–C(1) and H–C(9), between H–C(6) and Me(14), and between H–C(6) and Me(15) indicated that H–C(1) and H–C(4) were on the same side of the six-membered ring and in α-orientations, while H–C(6), Me(14), and Me(15) were β-orientated (*Fig. 2*). All NMR assignments were fully consistent with literature values for the structurally similar, known compound phomenone [5], phaseolinone [6], and xylarenal A [7]. Thus, from the above data, the structure of compound **1** was established as 6,7-epoxy-1,13-dihydroxyeremophila-9,11-dien-8-one, and named *xylarenone A*.

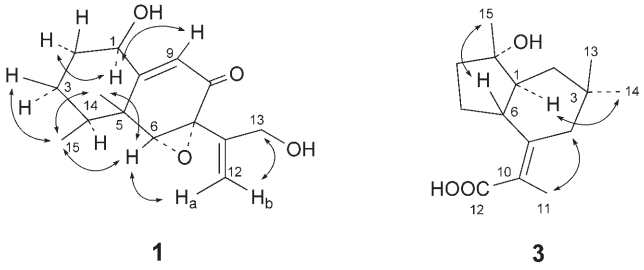

Fig. 2. *Key ROESY correlations for* **1** *and NOESY correlations for* **3**

Compound **2** was obtained as a colorless oil. The molecular formula was determined as $C_{17}H_{22}O_5$ on the basis of HR-Q-TOF-MS and NMR data. The NMR data of **2** (*Table 1*) were similar to those of **1**, except that **2** contained two more C-atoms at δ(C) 20.9 (C(17)) and 170.4 (C(16)), indicating an AcO group, which was located in 13-position, as inferred from $^1H,^{13}C$ long-range correlations between H–C(13) and C(16). The configuration of **2** was determined based on the same ROSEY correlations as in **1**.

From the above data, the structure of compound **2** was, thus, determined as 6,7-epoxy-1-hydroxy-8-oxoeremophila-9,11-dien-13-yl acetate, and named *xylarenone B.*

Compound **3** was obtained as a colorless oil. The molecular formula was determined as $C_{15}H_{24}O_3$ on the basis of HR-Q-TOF-MS and NMR data. The IR spectrum exhibited absorptions at 3415 cm^{-1} typical for OH groups. The ^1H- and ^{13}C-NMR spectra of **3** (*Table 2*), along with DEPT and HMQC experiments, revealed the signals of four Me

*singlet*s, four CH_2 and two CH groups, and five quaternary C-atoms, including two olefinic resonances at δ(C) 146.3 (C(5)) and 122.6 (C(10)), as well as a COOH moiety at δ(C) 175.3 (C(12)). Furthermore, the HMBC spectrum facilitated the assignments of all ^{1}H- and ^{13}C-NMR signals, thus indicating a bicyclic brasilane-type structure [8][9]. The HMBC correlation from Me(15) to C(1), C(8), and C(9), and from CH_2(8) to C(7), as well as from both H–C(1) and H–C(8) to C(6), established fragment **C** (*Fig. 1*). Additionally, the HMBC correlations from H–C(11) to C(5), C(10) and C(12), and from CH_2(4) to C(5) and C(10), along with the correlations of the H-atoms of both Me(13) and Me(14) to C(2), C(3) and C(4), respectively, led to the establishment of fragment **D** (*Fig. 1*). Finally, the HMBC correlations between H–C(1) and C(2), and between H–C(6) and C(4), connected fragments **C** and **D**.

Table 2. *^{1}H- and ^{13}C-NMR Data of* **3**. At 500/125 MHz, resp., in $CDCl_3$; δ in ppm, J in Hz.

Position	δ(H)	δ(C)
1	1.84 (*dd*, J = 12.8, 3.6)	53.3 (*d*)
2	1.11 (*t*, J = 12.8, H_α), 1.57 (*dd*, J = 12.8, 3.6, H_β)	38.5 (*t*)
3		33.3 (*s*)
4	2.48 (*d*, J = 14.1, H_α), 1.95–1.98 (*m*, H_β)	45.1(*t*)
5		146.3 (*s*)
6	2.00–2.02 (*m*)	48.9 (*d*)
7	2.06–2.09 (*m*, H_α), 1.88–1.90 (*m*, H_β)	28.1 (*t*)
8	1.90–1.92 (*m*, H_α), 1.70–1.73 (*m*, H_β)	41.7 (*t*)
9		77.6 (*s*)
10		122.6 (*s*)
11	2.00 (*s*)	16.1 (*q*)
12		175.3 (*s*)
13	0.97 (*s*)	32.0 (*q*)
14	0.93 (*s*)	27.1 (*q*)
15	1.18 (*s*)	25.7 (*q*)

The relative configuration of **3** was determined by NOESY experiments. The NOE correlations between H–C(6) and Me(15), and between H–C(1) and Me(14) indicated a *trans* fusion of rings *A* and *B* in **3**, in analogy to a related compound reported before [9]. Additionally, the NOE between H–C(4) and Me(11) helped to establish the (*Z*)-configuration of the $\Delta^{5(10)}$ double bond (*Fig. 2*).

From the above data, the structure of compound **3** was, thus, determined as 9-hydroxybrasil-5(10)-en-12-carboxylic acid[2]), and named *xylarenic acid*.

2. *Biological Properties.* Compounds **1**–**3** were tested in antitumor and antimicrobial assays *in vitro*. The results are collected in *Tables 3* and *4*. As can be seen, the new compounds exhibited moderate antitumor activities against HeLa cells (*Table 3*). In

[2]) For systematic names, see *Exper. Part*.

Table 3. *Growth-Inhibitory Effects of* **1**–**3** *against HeLa and HepG2 Cells.* The drugs were tested at 10 μg/ml; values are reported in percent (%) relative to blank control.

Compound	*MIC* [μg/ml]	
	HepG2	HeLa
1	8.7	27.8
2	23.8	21.1
3	2.63	19.9

Table 4. *Growth-Inhibitory Effects of* **1**–**3** *against Pathogenic Bacteria and Yeasts.* The drugs were tested at 50 μg/ml; values are reported in percent (%) relative to blank control.

Species	**1**	**2**	**3**
Escherichia coli	13.5	14.9	20.1
Bacillus subtilis	21.6	36.3	20.1
Staphylococcus aureus	21.6	36.3	23.5
Saccharomyces cerevisiae	1.13	2.56	0.39
Candida albicans	– 2.61	1.75	1.28

the antibacterial assay, compounds **1**–**3** inhibited the growth of bacteria, but had no effect on the growth of yeasts at a concentration of 50 μg/ml (*Table 4*).

The genus *Xylaria* is known for being a rich source of structurally diverse natural products, including polyketides [10], terpenoids [7][11][12], cyclopeptides [13], and unique unclassified xyloketals [14]. Among these compounds, one of the terpenoids, sordarin [15], is most interesting because of its significant antifungal activity by inhibiting the elongation factor of fungal protein synthesis [16]. One strain of this genus was reported to produce sordarin [12]. The present *Xylaria* sp. NCY2 strain was found to be rich in terpenoids, as demonstrated by the isolation of three new sesquiterpenoids belonging to two different structural types, eremophilane and brasilane. Notably, the brasilane derivative **3** (xylarenic acid), was first isolated from this genus. Our results, thus, call for further phytochemical and biological explorations of this fungal material and its potentially bioactive terpenoids.

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Experimental Part

General. Column chromatography (CC): silica gel (200–300 or 80–100 mesh; *Qingdao Marine Chemical Factory*, Qingdao, P. R. China), silica gel GF_{254} (*Merck*), *RP-18* gel (*Merck*), and *Sephadex LH-20* gel (*Amersham Biosciences*). Thin-layer chromatography (TLC): precoated silica-gel GF_{254} plates (0.20–0.25 mm; *Qingdao*). Optical rotations: *Perkin-Elmer-341* ploarimeter, in $CHCl_3$. IR Spectra: *Nicolet FT-IR 360* apparatus, KBr matrix; in cm^{-1}. ^1H- and ^{13}C-NMR Spectra: *Bruker DRX-500* spectrometer, at 500/125 MHz, resp., in $CDCl_3$; δ in ppm rel. to Me_4Si, J in Hz.

Isolation and Fermentation of the Fungal Strain. The fungus was isolated from *Torreya jackii* CHUN, collected in Jiangshi Nature Reserve Zone of Fujian Province, China, by the hyphal-tip method. Sequencing was performed according to both traditional morphology and internal transcribed spaces (ITS), which established that the fungus belongs to *Xylaria* species. Fermentation was performed, and

the mycelia of NCY2 grown on potato-dextrose agar (PDA) plates were used to inoculate 1-l *Erlenmeyer* flasks containing 200 ml of potato-dextrose (PD) medium (200 g/l potato, 20 g/l glucose, pH-neutral). The flasks were incubated on a rotary shaker for 5 d at 28° at 160 r.p.m. The cultures were then transferred into 16 20-l flasks containing PD medium (5 l), and cultivated for 6 months at 28° without agitation.

Extraction and Isolation. The culture filtrate was concentrated under vacuum at 45° to a volume of 20 l, and then extracted with AcOEt (3×). The combined org. layer, upon solvent removal, yielded a crude extract as a brown syrup (34.7 g). The extract was subjected to MPLC (140 g *RP-18*; H_2O, 30, 50, and 70% aq. acetone, neat acetone; 2 l for each solvent system) to yield 17 fractions: *Fr. 1 – Fr. 17. Fr. 3* (4.1 g) was separated by CC (*Sephadex LH-20*; MeOH) to afford three fractions: *Fr. 3.1 – Fr. 3.3*. Further separations by repeated CC (*RP-18*, acetone/H_2O; *Sephadex LH-20*, MeOH) yielded **1** (11 mg) and **3** (5 mg). Compound **2** (8 mg) was obtained from *Fr. 4* (350 mg) by CC (1. *RP-18*, MeOH/H_2O; 2. *Sephadex LH-20*, MeOH; 3. SiO_2, petroleum ether/acetone).

Xylarenone A (=*(Z)-6α,7α-Epoxy-1β,13-dihydroxyeremophila-9,11-dien-8-one*; **1**). Colorless oil. $[\alpha]_D^{20} = +144.1$ ($c = 4.610$, $CHCl_3$). IR (KBr): 3421. ^{1}H- and ^{13}C-NMR: see *Table 1*. ESI-MS (pos.): 287 ($[M+Na]^+$). HR-Q-TOF-MS: 287.1856 ($[M+Na]^+$, $C_{15}H_{20}NaO_4^+$; calc. 287.1259)

Xylarenone B (=*(Z)-6α,7α-Epoxy-1β-hydroxy-8-oxoeremophila-9,11-dien-13-yl Acetate*; **2**). Colorless oil. $[\alpha]_D^{20} = +62.7$ ($c = 0.614$, $CHCl_3$). IR (KBr) : 3443, 1740. ^{1}H- and ^{13}C-NMR: *see Table 1*. ESI-MS (pos.): 307 ($[M+H]^+$). HR-Q-TOF-MS: 329.2239 ($[M+Na]^+$, $C_{17}H_{22}NaO_5^+$; calc. 329.1365), 345.2017 ($[M+K]^+$, $C_{17}H_{22}KO_5^+$; calc. 345.1104).

Xylarenic Acid (=*9α-Hydroxybrasil-5(10)-en-12-carboxylic Acid*; *(Z)-2-(Octahydro-1-hydroxy-1,6,6-trimethyl-4*H*-inden-4-ylidene)propanoic Acid*; **3**). Colorless oil. $[\alpha]_D^{20} = -6.6$ ($c = 0.033$, $CHCl_3$). IR (KBr): 3415. ^{1}H- and ^{13}C-NMR: see *Table 2*. HR-Q-TOF-MS: 275.1698 ($[M+Na]^+$, $C_{15}H_{24}NaO_3^+$; calc. 275.1623).

Biological Studies. The cytotoxicities of compounds **1**–**3** were investigated by means of the MTT (= 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay [17] using the human cancer cell lines HepG2 and HeLa, cisplatin being used as pos. control. Further, the antibacterial activities of the new isolates were tested against three bacteria (*Escherichia coli*, *Bacillus subtilis*, and *Staphylococcus aureus*) and two yeasts (*Saccharomyces cerevisiae* and *Candida albicans*) by the minimal-inhibitory concentration (*MIC*) method [18] using 96-well microplates. Three replicates were performed for each compound at a concentration of 50 μg/ml.

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