Three New Triterpenes from *Xylarialean* sp. A45, an Endophytic Fungus from *Annona squamosa* L.

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From the fermentation extract of *Xylarialean* sp. A45, an endophytic fungus of *Annona squamosa* L., three new triterpenes, namely xylariacins A–C (**1**–**3**, resp.) were obtained. Their structures were determined by spectroscopic analyses, including 1D- and 2D-NMR experiments and HR-ESI-Q-TOF mass spectrometry. The *in vitro* cytotoxic activities of compounds **1**–**3** were tested against human tumor cell line HepG2, and these compounds showed modest cytotoxic activity.

Introduction. – Recently, increasing attention has been paid to plant commensal microorganisms as a source of new bioactive substances [1–3]. Especially, there are several trees whose endophytes produce various bioactive secondary metabolites.

Many members of the Annonaceae are used in folk medicine for antiparasitic or antitumoral treatment of intestinal diseases [4]. Among them, *Annona squamosa* L. produces many kinds of Annonaceous acetogenins which showed cytotoxicities against several human cancer cell lines. They are now of great interest for their antitumor properties [5]. In recent years, many novel structures and a wide range of bioactivities were discovered in this tree [6]. Endophytes, as a major part of this tree's ecosystem, play an important role in producing bioactive compounds. Due to the long term of coevolution, endophytes from this plant may possess abundant and novel secondary metabolites.

During the course of our search for new bioactive compounds from the plant endophytic fungus *Xylarialean* sp. A45, three new triterpenes, **1**–**3** (*Fig.*), were purified and elucidated. Here, we report their isolation, structure elucidation and the cytotoxic activities.

Results and Discussion. – Strain A45 was fermented for 16 d at 28° on PDA (Potato Dextrose Agar) media (10 l). The AcOEt extract of fermentation was chromatographed repeatedly on various columns and yielded the three compounds **1**–**3**.

Compound **1** was obtained as white powder, and the HR-Q-TOF-MS showed a *pseudo*-molecular-ion peak at *m*/*z* 513.3766 ($[M+Na]^+$; calc. 513.3294) corresponding to the molecular formula $C_{29}H_{46}O_6$, with seven degrees of unsaturation.

The ^{13}C-NMR (DEPT) spectra of compound **1** revealed 29 signals: for eight Me, six CH_2, seven CH groups, and eight quaternary C-atoms.

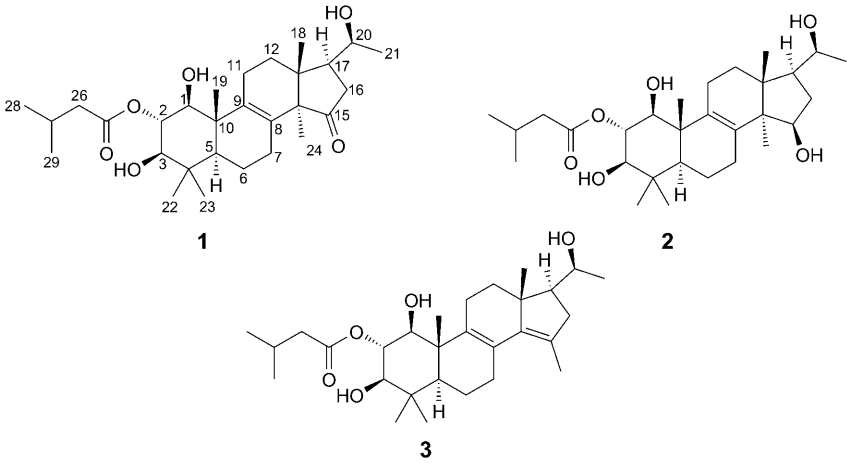

Figure. *Structures of compounds* **1**, **2**, *and* **3**

The structure of compound **1** was elucidated on the basis of the following evidence. The ^{1}H-NMR spectrum of compound **1** (*Table*) exhibited five *singlet*s at δ(H) 0.75, 1.08, 0.91, 1.05, and 1.10, which were assigned to the Me(18), Me(19), Me(22), Me(23), and Me(24), respectively, and three *doublet*s at δ(H) 1.30 ($J = 5.6$), 1.01, and 1.01 ($J =$ 7.2) were assigned to the Me(21), Me(28), and Me(29) groups, respectively. Four characteristic downfield signals at δ(H) 3.23 ($d, J = 10.2$), 3.52 ($d, J = 6.7$), 3.83 ($dq, J =$ 6.2, 7.9), and 4.97 (t, $J = 10.0$) were due to H-atoms geminal to an O-bearing substituent.

The ^{13}C-NMR and DEPT spectra of **1** showed resonances for 29 C-atoms. The downfield resonances at δ(C) 175.6 and 215.9 were assigned to the C(25) and C(15) CO C-atoms, respectively. Four CH signals at δ(C) 69.7, 77.4, 77.7, and 78.1 were assigned to O-bearing C-atoms C(20), C(1), C(2), and C(3), respectively. The resonances at δ(C) 15.2, 15.9, 16.2, 22.4, 22.4, 24.2, 25.9, and 28.1 were ascribed to the Me groups (Me(19), Me(22), Me(18), Me(28), Me(29), Me(21), Me(27), and Me(23), resp.). The overall NMR data were in agreement with the known compound 4,4,14-trimethyl-5α-pregn-8-ene-3β,20β-diol [7] and one 3-methylbutanoic acid moiety. Unambiguous assignments of the ^{1}H- and ^{13}C-NMR signals were achieved by combination of DEPT, HSQC, and HMBC. From the analysis mentioned above, the chemical structure of compound **1** was identified as (1β,2α,3β,5α,20*S*)-1,3,20-trihydroxy-4,4,14-trimethyl-15-oxopregn-8-en-2-yl 3-methylbutanoate (*Fig.*), named xylariacin A.

Compound **2** was obtained as a white powder, and its molecular formula was determined as $C_{29}H_{48}O_6$ by HR-Q-TOF-MS (positive-ion mode; $[M + Na]^+$ at m/z 515.3789; calc. 515.3451) and ^{13}C-NMR. The structure of **2** was elucidated to be a 15-hydroxy derivative of **1** by comparison of NMR spectra with those of **1** (*Table*). In **1**, C(15) (δ(C) 215.9) was a ketone group, whereas, in **2**, C(15) (δ(C) 73.7) was a OH-substituted CH group. Therefore, the chemical structure of compound **2** was determined as shown and named xylariacin B.

Table. *^{13}C- and ^{1}H-NMR Data of* **1**, **2**, *and* **3**. Recorded at 150 and 600 MHz, resp., in MeOD; δ in ppm, J in Hz.

	1		**2**		**3**	
	δ(C)	δ(H)	δ(C)	δ(H)	δ(C)	δ(H)
H–C(1)	77.4	3.52 (*d*, *J* = 10.0)	76.1	3.53 (*d*, *J* = 9.8)	76.0	3.61 (*d*, *J* = 9.5)
H–C(2)	77.7	4.97 (*t*, *J* = 10.6)	77.7	4.97 (*dd*, *J* = 9.6, 10.6)	77.7	5.00 (*dd*, *J* = 9.6, 10.0)
H–C(3)	78.1	3.23 (*d*, *J* = 10.2)	78.2	3.23 (*d*, *J* = 10.4)	78.0	3.24 (*d*, *J* = 10.3)
C(4)	39.2	–	39.2	–	39.2	
H–C(5)	47.0	1.08 (*m*, overlapped)	47.5	1.12 (*dd*, *J* = 8.8, 9.1)	47.1	1.12 (*t*, *J* = 7.1)
CH_2(6)	17.4	1.03 – 1.09 (*m*)	17.1	1.00 – 1.02 (*m*)	18.3	0.99 – 1.02 (*m*)
CH_2(7)	25.8	2.73 – 2.75, 2.37 – 2.40 (2*m*)	26.7	2.16 – 2.20 (*m*)	25.2	2.38 – 2.42 (*m*)
C(8)	132.5	–	134.1	–	125.1	–
C(9)	136.5	–	135.5	–	141.0	–
C(10)	44.1	–	44.8	–	44.9	–
CH_2(11)	23.6	2.49 (*dd*, *J* = 7.4, 9.5), 2.24 (*d*, *J* = 9.5)	23.9	2.42 (*dd*, *J* = 10.2, 9.0), 2.16 (*dd*, *J* = 10.2, 9.0)	31.0	2.40 – 2.43 (*m*)
CH_2(12)	28.9	1.84 (*d*, *J* = 8.7), 1.55 – 1.60 (*m*)	30.9	1.79 (*dd*, *J* = 11.4, 10.6), 1.58 (*dd*, *J* = 11.4, 10.6)	36.9	1.86 – 1.90, 1.32 – 1.37 (2*m*)
C(13)	57.1	–	51.5	–	46.3	–
C(14)	42.0	–	43.4	–	128.9	–
C(15) or H–C(15)	215.9	–	73.7	4.29 (*dd*, *J* = 9.3, 5.2)	139.9	–
CH_2(16)	39.8	2.67 (*dd*, *J* = 8.6, 8.1), 2.27 (*dd*, *J* = 10.3, 9.5)	37.0	2.27 (*d*, *J* = 6.7), 1.77 (*d*, *J* = 5.5)	42.6	2.51 (*d*, *J* = 6.7)
H–C(17)	48.0	1.98 (*dt*, *J* = 9.7, 8.3)	51.4	1.76 – 1.82 (*m*)	56.7	1.61 – 1.65 (*m*)
Me(18)	16.2	0.75 (*s*)	16.5	0.71 (*s*)	15.8	0.76 (*s*)
Me(19)	15.2	1.08 (*s*)	15.4	1.11 (*s*)	16.4	1.15 (*s*)
H–C(20)	69.7	3.83 (*dq*, *J* = 6.2, 7.9)	70.6	3.68 (*dq*, *J* = 5.9, 6.4)	69.4	3.89 (*q*, *J* = 6.7)
Me(21)	24.2	1.30 (*d*, *J* = 5.6)	23.6	1.21 (*d*, *J* = 5.9)	23.8	1.25 (*d*, *J* = 6.0)
Me(22)	15.9	0.91 (*s*)	16.0	0.91 (*s*)	16.0	0.92 (*s*)
Me(23)	28.1	1.05 (*s*)	28.1	1.04 (*s*)	28.1	1.06 (*s*)
Me(24)	21.8	1.10 (*s*)	21.8	1.10 (*s*)	17.6	1.92 (*s*)
C(25)	175.6	–	175.5	–	175.6	–
CH_2(26)	43.6	2.34 (*d*, *J* = 7.4)	43.7	2.31 (*d*, *J* = 7.3)	43.6	2.32 (*d*, *J* = 7.1)
H–C(27)	25.9	2.12 – 2.17 (*m*)	25.9	2.11 – 2.17 (*m*)	25.9	2.15 – 2.19 (*m*)
Me(28)	22.4	1.01 (*d*, *J* = 7.2)	22.4	1.00 (*d*, *J* = 6.5)	22.4	1.02 (*d*, *J* = 6.6)
Me(29)	22.4	1.01 (*d*, *J* = 7.2)	22.4	1.00 (*d*, *J* = 6.5)	22.4	1.02 (*d*, *J* = 6.6)

Compound **3** was isolated as white needles, and its molecular formula was determined as $C_{29}H_{46}O_5$ by positive-ion HR-Q-TOF-MS ($[M+Na]^+$ at m/z 497.3878; calc. 497.3345) and ^{13}C-NMR. The ^{13}C-NMR (DEPT) spectra (*Table*) of **3** were similar to those of **1**, except that **3** did not have a C(15)=O group, C(14) and C(15) of **3** were connected with a C=C bond with a Me group at C(15), which was confirmed by the HMBCs from a Me group at δ(H) 1.92 (Me(24)) to C(14), C(15), and C(16). The chemical structure was elucidated as (1β,2α,3β,5α,20*S*)-1,3,20-trihydroxy-4,4,15-trimethylpregna-8,14-dien-2-yl 3-methylbutanoate, named xylariacin C.

The cytotoxic activity against HepG-2 cell was tested by the MTT (= 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2*H*-tetrazolium bromide) method [8]. The inhib-

itory rates of compounds **1**–**3** was 48.0, 9.7, and 46.7%, respectively, at the concentration 20 μg/ml.

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Experimental Part

General. Column chromatography (CC): silica gel (SiO_2; 200–300 mesh; *Qingdao Marine Chemical, Inc.*, Qingdao, P. R.China), silica gel *H* (10–40 μm, Qingdao), *Sephadex LH-20* (4–70 μm, *Amersham Pharmacia Biotech AB*, Uppsala, Sweden), and *281 Lichroprep* reversed-phase *RP-18* silica gel (40–63 μm, *Merck*, Darmstadt, Germany). Prep. TLC (1.0–1.5 mm): glass precoated with silica gel GF_{254} (*Qingdao*), and visualization under UV light. Optical rotations: *Perkin-Elmer 341* automatic polarimeter, in MeOH. IR Spectra: *Nicolet AVATAR 330 FT* spectrometer; in cm^{-1}; in KBr pellets. NMR Spectra: *Bruker DRX-600* NMR spectrometer with TMS as an internal standard. HR-Q-TOF-MS and ESI-MS: *VG Auto-Spec-3000* and *Bruker BioTOF-Q*, resp.

Isolation and Fermentation of the Fungal Strain. The fungus was isolated from the current-year phloem of *Annona squamosa* L. collected from the Xiamen University, Fujian, P. R. China by the hyphal-tip method [9]. Sequencing was performed according to both traditional morphology and internal transcribed spaces (ITS), which established that the fungus belongs to *Xylarialean* species. Fermentation was performed for 16 d at 28° on PDA (potato dextrose agar) media (10 l).

Extraction and Isolation. The culture media was collected and extracted three times with AcOEt/MeOH/AcOH 80:15:5 at r.t. overnight. The org. soln. was collected through filtration. The combined filtrates were concentrated under vacuum to remove org. solvents. The aq. soln. was extracted with AcOEt. The combined org. layer, upon solvent removal, yielded a crude extract (3.6 g).

Isolation. The extract was subjected to MPLC (80 g *RP-18*; H_2O, 30, 50, and 70% aq. MeOH, neat MeOH; 2 l for each solvent system) to yield eight fractions: *Frs. 1–8*. These fractions were further purified by repeated CC (*Sephadex LH-20* and SiO_2).

Fr. 6 (260 mg) was separated by CC (*Sephadex LH-20* (140 g); MeOH) to give a subfraction (*Fr. 6a*). *Fr. 6a* (38.4 mg) was further purified by CC (SiO_2; PE/AcOEt 20:1; 18:1; 15:1; 12.5:1 (*v*/*v*)) to give *Fr. 6a1*. *Fr. 6a1* was separated by CC (*RP-18* silica gel (30 g): MeOH/H_2O 40:60, 50:50, 60:40, and 100:0 (*v*/*v*)) to yield *Fr. 6a1a*. *Fr. 6a1a* was separated by CC (*Sephadex LH-20* (140 g); MeOH) to afford a subfraction (*Fr. 6a1a1*). *Fr. 6a1a1* was further purified by CC (SiO_2; PE/AcOEt 14:1 (*v*/*v*)) to yield **3** (5.9 mg). *Fr. 5* (890 mg) was separated by CC (*Sephadex LH-20* (140 g); MeOH) to three subfractions, *Frs. 5a–5c*. *Fr. 5a* (180 mg) was further purified by CC (SiO_2; PE/AcOEt 20:1; 10:1; 8:1; 5:1; 3:1; 0:1 (*v*/*v*)) to give two subfractions, *Frs. 5a-2* and *5a-3*. *Fr. 5a-2* (34 mg) was separated into two fractions, *Frs. 5a-2-A* and *5a-2-C* by CC (*Sephadex LH-20* (140 g); acetone). *Fr. 5a-2-A* was further purified by CC (SiO_2; PE/AcOEt 6:1; 5:1; 4:1; 3:1; 2:1 (*v*/*v*)) to yield **1** (4.5 mg). *Fr. 5a-3* (7.6 mg) was further purified by CC (SiO_2; PE/AcOEt 10:1; 8:1; 7:1; 6:1; 4:1 (*v*/*v*)) to afford *Fr. 5a-3-a*, which was separated by CC (*Sephadex LH-20* (80 g); acetone) to give **2** (1.7 mg).

Xylariacin A (=*(1β,2α,3β,5α,20*S*)-1,3,20-Trihydroxy-4,4,14-trimethyl-15-oxopregn-8-en-2-yl 3-Methylbutanoate*; **1**). Powder. $[\alpha]_D^{20} = +1.6$ ($c = 0.5$, MeOH). IR (KBr): 3452, 2975, 1378. ^{1}H- and ^{13}C-NMR: see the *Table*. ESI-MS: 513.1 ($[M+Na]^+$). HR-Q-TOF-MS: 513.3766 ($[M+Na]^+$; calc 513.3294).

Xylariacin B (=*(1β,2α,3β,5α,15β,20*S*)-1,3,15,20-Tetrahydroxy-4,4,14-trimethylpregn-8-en-2-yl 3-Methylbutanoate*; **2**). White powder. $[\alpha]_D^{20} = +2$ ($c = 0.5$, MeOH). IR (KBr): 3398, 2963, 1631, 1465. ^{1}H- and ^{13}C-NMR: see the *Table*. ESI-MS: 515.1 ($[M+Na]^+$). HR-Q-TOF-MS: 515.3789 ($[M+Na]^+$; calc 515.3451).

Xylariacin C (=*(1β,2α,3β,5α,20*S*)-1,3,20-Trihydroxy-4,4,15-trimethylpregna-8,14-dien-2-yl 3-Methylbutanoate*; **3**). White needles. $[\alpha]_D^{20} = +3.6$ ($c = 0.5$, MeOH). IR (KBr): 3352, 2921, 1602, 1374. ^{1}H- and ^{13}C-NMR: see the *Table*. ESI-MS: 497.1 ($[M+Na]^+$). HR-Q-TOF-MS: 497.3878 ($[M+Na]^+$; calc 497.3345), 513.3713 ($[M+K]^+$; calc 513.3545).

Biological Assay. The cytotoxicities of compounds **1**–**3** were tested by means of the MTT (= 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2*H*-tetrazolium bromide) assay [8] using the human cancer cell lines HepG2, cisplatin being used as positive control.

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