A New Peroxy-multiflorane Triterpene Ester from the Processed Seeds of *Trichosanthes kirilowii*

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A new peroxy-multiflorane triterpene ester, (3*α*,5*α*,8*α*,20*α*)-5,8-epidioxymultiflora-6,9(11)-diene-3,29-diol 3,29-dibenzoate (**1**), was isolated from the processed seeds of *Trichosanthes kirilowii*, together with the two known related derivatives **2** and **3**, and the two known steroids **4** and **5**. Compounds **2**, **4**, and **5** were isolated from the genus *Trichosanthes* for the first time. The structure of compound **1** was established by NMR, HR-MS, and CD analyses. Compounds **1**–**3** were tested for their *in vitro* cytotoxicity against human-tumor cell lines (Hela, HL-60, and MCF-7) and anti-inflammatory activity (LPS-induced B lymphocyte cells) with the MTT method.

Introduction. – The seeds of *Trichosanthes kirilowii* MAXIM. (Cucurbitaceae), are used as traditional Chinese medicine for the treatment of inflammation, cough, and phlegm [1]. Species of *Trichosanthes* are well-known for their production of biologically active triterpenes especially of the multiflorane type [2] (multiflorane = *D:C*-friedooleanane = (13*α*,14*β*)-13-methyl-26-noroleanane). This type of triterpenes showed an inhibitory effect against EBV-EA activation and antitumor-promoting effects, some of them exhibited cytotoxic activity against human-cancer cell lines [3], and marked inhibitory activity against 12-*O*-tetradecanoylphorbol 13-acetate (TPA) induced ear inflammation in mice [4].

For certain clinical applications, the seeds need to be processed by moderate heating and pressing to reduce the lapactic effect caused by fatty oil [5]. To obtain active compounds, an investigation of the extract of processed *Trichosanthes kirilowii* seeds was carried out, and a new peroxy-multiflorane triterpene ester, (3*α*,5*α*,8*α*,20*α*)-5,8-epidioxymultiflora-6,9(11)-diene-3,29-diol 3,29-dibenzoate (**1**), and two known related derivatives, karouni-3,29-diol 3,29-dibenzoate (**2**) [6] and (3*α*)-7-oxomultiflor-8-ene-3,29-diol 3,29-dibenzoate (**3**) [2], as well as two known steroids, (3*β*,5*α*,8*α*,22*E*)-5,8-epidioxyergosta-6,22-dien-3-ol (**4**) and (3*β*,5*α*,8*α*,22*E*)-5,8-epidioxyergosta-6,9(11),22-trien-3-ol (**5**) [7] (*Fig. 1*) were obtained. Compounds **2**, **4**, and **5** were isolated from the genus *Trichosanthes* for the first time. Herein, we describe the isolation and structure elucidation of compound **1**, and the result of the *in vitro* cytotoxicity and anti-inflammatory activity test of **1**–**3**.

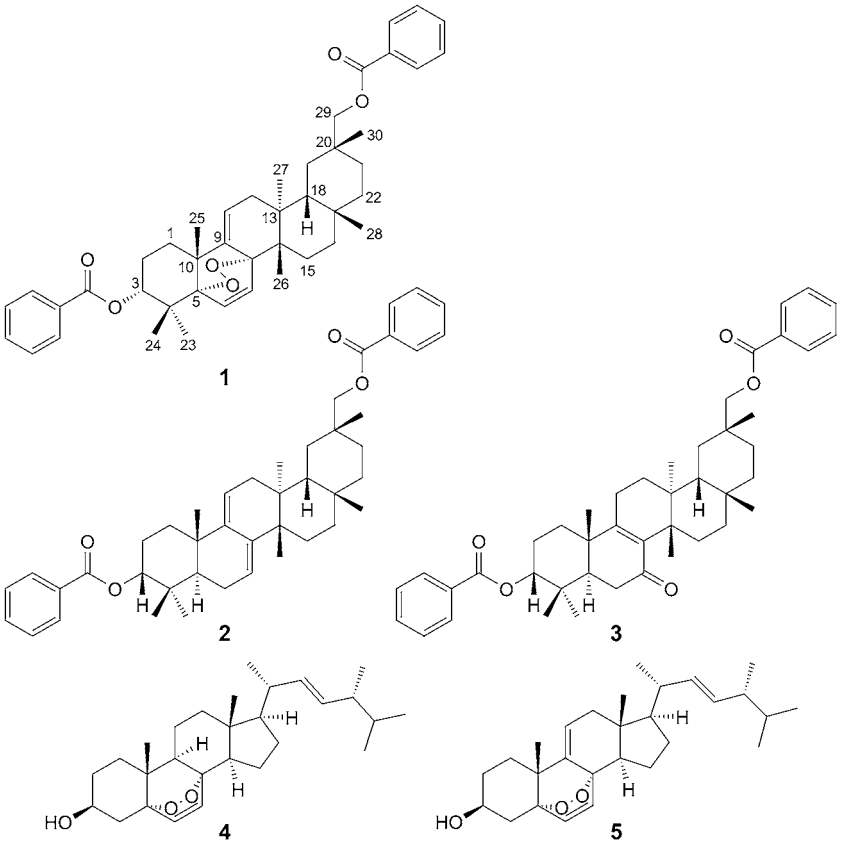

Fig. 1. *Compounds* **1**–**5**, *isolated from the processed seeds of* Trichosanthes kirilowii

Results and Discussion. – Compound **1** was obtained as a white amorphous solid, and its molecular formula, $C_{44}H_{54}O_6$, was deduced from HR-ESI-TOF-MS data (*m*/*z* 701.3817, $[M+Na]^+$) and NMR data. The IR absorptions at 1717, 1602, 1452, and 713 cm^{-1}, respectively, provided evidence of C=O and Ph groups. The ^{1}H-NMR spectrum (*Table*) of **1** indicated the presence of seven Me groups (7*s* at δ(H) 1.07, 1.12, 1.15, 1.17, 1.21, 1.24, and 1.30), one CH–O at δ(H) 4.95 (*t*, $J = 2.4$ Hz) and one CH_2O at 4.10 (*d*, $J = 10.8$ Hz, 2 H), three olefinic H-atoms at δ(H) 6.54 (*d*, $J = 9.0$ Hz, 1 H), 6.84 (*d*, $J = 9.0$ Hz, 1 H), and 5.51 (*dd*, $J = 6.3$, 1.5 Hz, 1 H), as well as the signals of ten aromatic H-atoms at δ(H) 7.43–8.17. The ^{13}C-NMR displayed twelve aromatic signals, two C=O signals, and 30 signals for the triterpene core, including four olefinic C-atoms at δ(C) 142.7, 131.9, 133.7, and 120.2, as well as four signals due to O-bearing C-atoms at δ(C) 85.5, 79.5, 76.2, and 73.8. These observations indicated that **1** was a tetraoxy-substituted triterpenoid carrying two benzoyl groups. The O-bearing C-atoms were determined to be in positions 3, 29, 5, and 8, as deduced from HMBCs between Me(23), Me(24) and C(3), C(5), between Me(30) and C(29), and between Me(26) and C(8),

Table. *^{1}H- and ^{13}C-NMR Data* (600 and 75 MHz, resp.; $CDCl_3$) *of* **1**. δ in ppm, J in Hz.

	δ(H)	δ(C)
CH_2(1)	1.53–1.55 (*m*), 2.43 (*td*, *J* = 13.8, 3.0)	29.7
CH_2(2)	1.88 (*dq*, *J* = 15.2, 3.0), 2.09 (*tt*, *J* = 14.5, 3.0)	23.2
H–C(3)	4.95 (*t*, *J* = 2.4)	76.2
C(4)		39.3
C(5)		85.5
H–C(6)	6.54 (*d*, *J* = 9.0)	131.9
H–C(7)	6.84 (*d*, *J* = 9.0)	133.7
C(8)		79.5
C(9)		142.7
C(10)		43.5
H–C(11)	5.51 (*dd*, *J* = 6.3, 1.5)	120.2
CH_2(12)	1.76–1.77 (*m*), 1.97 (*dd*, *J* = 17.5, 6.6)	38.0
C(13)		38.1
C(14)		40.3
CH_2(15)	1.42–1.44 (*m*), 2.18 (*dt*, *J* = 13.8, 3.0)	24.4
CH_2(16)	1.48–1.50, 1.58–1.62 (2*m*)	35.4
C(17)		31.8
H–C(18)	1.71–1.73 (*m*)	44.3
CH_2(19)	1.35–1.37, 1.78–1.79 (2*m*)	29.4
C(20)		32.1
CH_2(21)	1.28, 1.53–1.55 (2*m*)	29.2
CH_2(22)	0.97–0.99, 1.67–1.69 (2*m*)	35.5
Me(23)	1.07 (*s*)	23.1
Me(24)	1.30 (*s*)	25.0
Me(25)	1.17 (*s*)	27.3
Me(26)	1.21 (*s*)	22.7
Me(27)	1.24 (*s*)	20.3
Me(28)	1.15 (*s*)	30.1
CH_2(29)	4.10 (*d*, *J* = 10.8)	73.8
Me(30)	1.12 (*s*)	28.6
*C*OO–C(29)		166.3
C(1′)		130.6
H–C(2′,6′)	8.17 (br. *d*, *J* = 8.4, 2 H)	130.0
H–C(3′,5′)	7.43 (*t*, *J* = 7.8, 2 H)	128.4
H–C(4′)	7.52 (br. *t*, *J* = 7.4)	132.7
*C*OO–C(3)		166.7
C(1″)		130.6
H–C(2″,6″)	8.05 (br. *d*, *J* = 8.4, 2 H)	129.5
H–C(3″,5″)	7.45 (*t*, *J* = 7.8, 2 H)	128.4
H–C(4″)	7.56 (br. *t*, *J* = 7.4)	132.8

respectively. The correlations between H–C(3) and *C*OO–C(3), and between CH_2(29) and *C*OO–C(29) suggested the 3,29-dibenzoylation. The molecular formula indicated that the remaining two O-atoms should be in an endoperoxide moiety. This was located at C(5) and C(8) according to the HMBCs between Me(23), Me(24), and H–C(6) and C(5) as well as between H–C(7) and Me(26) and C(8). Comparison of the chemical shifts of C(5) (δ(C) 85.5) and C(8) (δ(C) 79.5) with those observed in compounds **4** and **5** (δ(C(5)) 82.1 (**4**) and 82.7 (**5**); δ(C(8)) 79.4 (**4**) and 78.4 (**5**)) provided additional

evidence. The C=C bonds were placed between C(6) and C(7), and between C(9) and C(11) based on the correlations H–C(6)/C(4) and C(5), H–C(7)/C(8) and C(9), as well as Me(25)/C(9) (*Fig. 2*).

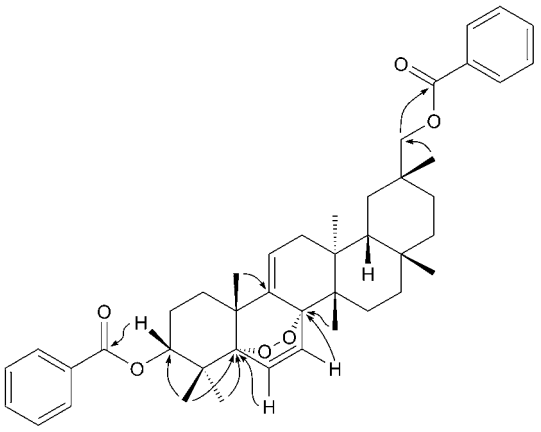

Fig. 2. *Selected HMBCs of compound* **1**

The relative configuration of **1** was determined by the NOESY correlations (*Fig. 3*). The correlations between δ(H) 1.30 (Me(24)) and δ(H) 1.17 (Me(25)), as well as δ(H) 6.84 (H–C(7)) and δ(H) 1.21 (Me(26)) indicated that these Me groups were β-orientated, further demonstrating the α-orientation of the epidioxy group. The correlation between δ(H) 4.95 (H–C(3)) and δ(H) 1.30 (Me(24)) suggested the α-orientation of the benzoyloxy substitution at C(3). The correlation between δ(H) 4.10 (CH_2(29)) and δ(H) 1.24 (Me(27)) suggested that they were α-orientated. The CD spectrum of **1** showed a positive *Cotton* effect at 276 nm (*Fig. 4*) which is consistent with the axiom of the exciton chirality method (*Fig. 5*) [8][9]. The structure of **1** was

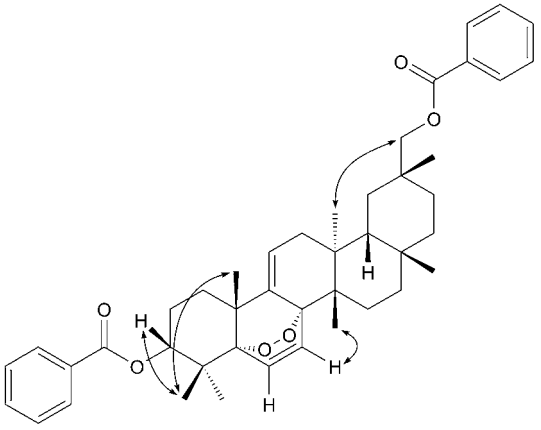

Fig. 3. *Selected NOESY correlations of compound* **1**

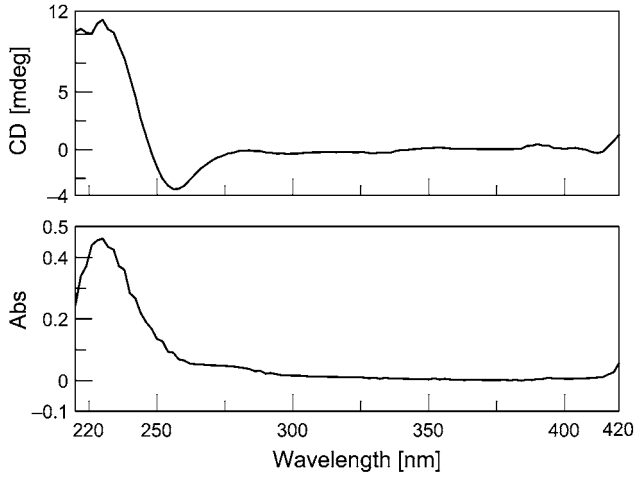

Fig. 4. *CD and UV Curves of compound* **1**

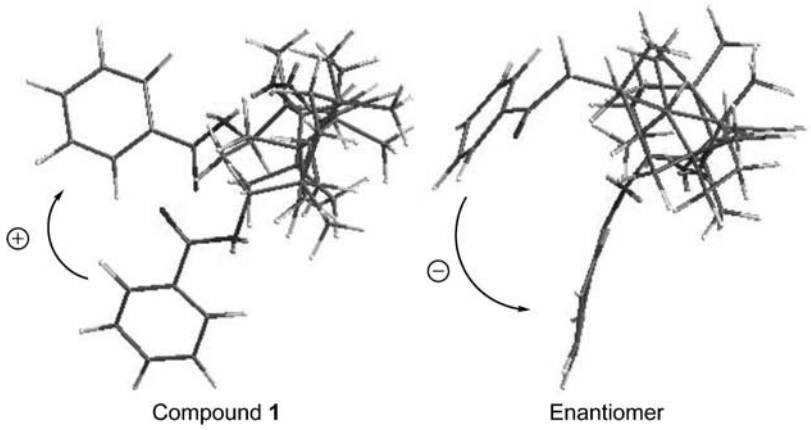

Fig. 5. *The* Cotton *effect of compound* **1** *and its enantiomer*

therefore elucidated as (3α,5α,8α,20α)-5,8-epidioxymultiflora-6,9(11)-diene-3,29-diol 3,29-dibenzoate.

Compound **1** was detected in both processed and unprocessed seeds by HPLC/MS analysis (see *Exper. Part*). This confirmed that compound **1** is not produced during the processing of the seeds. By means of the MTT (2-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2*H*-tetrazolium bromide) method, the *in vitro* cytotoxic and anti-inflammatory activity of compound **1** was assayed, together with those of **2** and **3**; compounds **1**–**3** showed no marked cytotoxicities against the Hela, HL-60, and MCF-7 cell lines and had no marked inhibitory activity towards LPS-induced B lymphocyte cells proliferation at 0.1–100 μM.

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Experimental Part

General. Column chromatography (CC): silica gel (SiO_2; 200–300 mesh; *Qingdao Haiyang Co.*, P. R. China). Prep. HPLC: *Hitachi-L-7110* pump, *Hitachi-L-7420* UV spectrophotometric detector at 210 nm, and *YMC-C_{18}* reversed-phase column (5 μm; 10 × 250 mm); flow rate 2.0 ml/min. CD Spectrum: *Jasco CD-2095-plus*; MeOH soln.; in nm (mdeg). UV Spectra: *Shimadzu-UV-2201* spectrophotometer; MeOH soln.; λ_{max} (log ε) in nm. IR Spectra: *IFS-55* spectrophotometer; KBr pellets; in cm^{-1}. NMR Spectra: *Bruker-AV-600* and *-ARX-300* spectrometer; δ in ppm rel. to Me_4Si as internal standard, *J* in Hz. ESI-MS: *Finnigan-LCQ-Deca-XP-MAX* spectrometer; in *m/z*. HR-TOF-MS: *Bruker-micro-TOF-Q-125* mass spectrometer; in *m/z*.

Plant Material. The seeds of *Trichosanthes kirilowii* MAXIM. were collected in Laiwu City, Shandong Province, P. R. China, in October, 2007, and authenticated by Prof. *Qishi Sun*, Shenyang Pharmaceutical University. A voucher specimen (No. 20071013) was deposited with the Research Department of Natural Medicine, Shenyang Pharmaceutical University. The seed coats were separated, and the obtained kernels were heated (50°) and pressed (20 min) after crushing to partially remove the fatty oil yielding the processed product (frost-like powder).

Extraction and Isolation. The processed product of *T. kirilowii* seeds (5.0 kg) was extracted with 95% EtOH (3 × 40 l, 3 h each time) under reflux conditions to give a crude extract (103.7 g). The extract was suspended in H_2O (5 l) and extracted successively with petroleum ether (3 × 5 l), $CHCl_3$ (3 × 5 l), and BuOH (3 × 5 l) to yield a petroleum ether-soluble fraction (30.7 g), a $CHCl_3$-soluble fraction (21.8 g), and a BuOH-soluble fraction (20.1 g). A part of the $CHCl_3$-soluble fraction (20.0 g) was subjected to CC (SiO_2, gradient petroleum ether/acetone): **2** (with 100:1; 28 mg), *Frs. A* (with 100:3; 32 mg), and *B* (with 100:5; 25 mg). *Frs. A* and *B* were further separated by reversed-phase HPLC (*ODS* column (250 × 10 mm), flow rate 2 ml/min): **1** (with 99% MeOH; t_R 32.5 min; 16 mg) and **3** (with 97% MeOH; t_R 23.2 min; 12 mg). A part of the petroleum ether-soluble fraction (30.0 g) was subjected to CC (SiO_2; gradient petroleum ether/acetone): *Fr. C* (with 100:1; 120 mg). *Fr. C* was further separated by prep. reversed-phase HPLC: **4** (with 99% MeOH; t_R 29.7 min; 11 mg) and **5** (with 99% MeOH; t_R 23.2 min; 9 mg).

Detection of **1** *in Unprocessed Seeds by HPLC/MS.* Extract: $CHCl_3$/MeOH 1:1. *Waters-Alliance-2695* seprations module and *LCT-Premier-XE* mass spectrometer; *Scienhome-Kromasil-ODS* column (5 μm; 250 mm × 4.6 mm i.d.); eluent MeOH (100%); flow 1 ml/min; 30°; detection between 100 and 1500 Da; compound **1**: t_R 11.12 min, $[M+Na]^+$ at *m/z* 701.3810.

(3α,5α,8α,20α)-5,8-Epidioxymultiflora-6,9(11)-diene-3,29-diol 3,29-Dibenzoate (= *(3α,8α,13α,14β,20α)-5,8-Epidioxy-13-methyl-26-noroleana-6,9(11)-dien-3,29-diol 3,29-Dibenzoate*; **1**): White amorphous solid ($CHCl_3$). $[\alpha]_D^{20} = -23.5$ ($c = 0.20$, $CHCl_3$). UV (MeOH): 228 (2.15). CD (MeOH): 220 (+10.20), 230 (+11.23), 256 (−3.44), 266 (−1.67), 276 (+0.04), 278 (−0.12). IR (KBr): 2920, 1717, 1602, 1452, 1276, 713. ^{1}H- and ^{13}C-NMR: *Table.* HR-TOF-MS: 701.3817 ($[M+Na]^+$, $C_{44}H_{54}NaO_6^+$; calc. 701.3813).

Cytotoxicity Assay. The Hela and HL-60 cell lines were cultured in *RPMI1640* in 96-well micro-culture plates, and the MCF-7 cell lines were cultured in *Dulbecco*'s modified *Eagle* medium. Compounds **1**–**3** were added in each well (in triplicate), the concentrations were 100, 10, 1, and 0.1 μM. Control cells received 5-fluorouracil (25 μg/ml). After incubation at 37° in a 5% CO_2 atmosphere for 72 h, MTT (15 μl) was added to each well. After incubation for another 4 h, the color change of the soln. was measured with a micro-autoreader at 490 nm. The cytotoxicity was calculated according to the IC_{50} value.

In vitro *Anti-inflammatory-Activity Assay.* The mice spleen was cut into pieces in *D-Hanks* soln. and ground (200 mesh sieve). The spleen cells were collected, and *Tris*-NH_4Cl solution was added to crack red blood cell. After centrifugation (1000 rpm for 10 min), the cells were washed by *D-Hanks* soln. (3×). Then, the cells were suspended in 10% FBS *RPIM1640* cell culture medium, the cell concentration

adjusted to $3 \cdot 10^6$/ml, and the suspension cultured in 96-well micro-culture plates. Compound **1**–**3** were added in each well (in triplicate), the concentrations were 100, 10, 1, and 0.1 μM. LPS was added (50 μl) to each well. After incubation at 37° in a 5% CO_2 atmosphere for 24 h, drop the supernatant (100 μl) slightly, MTT (15 μl) was added to each well. After incubation for another 4 h, the color change of the soln. was measured with a micro-autoreader at 490 nm. The inhibitory activity of LPS-induced B-lymphocyte proliferation was calculated according to the inhibitory rate [10].

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