FULL PAPER

Paeonenoides D and E: Two New Nortriterpenoids from *Paeonia lactiflora* and Their Inhibitory Activities on NO Production

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Two new nortriterpenoids, paeonenoides D and E (**1** and **2**, resp.), together with seven known compounds, were isolated from the roots of *Paeonia lactiflora*. Their structures were elucidated on the basis of spectroscopic evidence. Compounds **1**–**7** were screened for inhibitory effects against NO production in LPS-induced RAW246.7 macrophages and for cytotoxic activities against HL-60, Hep-G2, and SK-OV-3 cell lines. Compounds **1**–**3** and **5**–**7** exhibited inhibitory activities with IC_{50} values in the range of 9.6–32.2 μM. Triterpenoids with an epoxide ring and a free COOH function, **1**–**3**, showed effectively increased activities compared with other pentacyclic triterpenoids. Compounds **1**–**6** showed significant cytotoxic activities against the Hep-G2 cell line and modest cytotoxic activities against HL-60 and SK-OV-3 cell lines.

Introduction. – *Paeonia lactiflora* Pall. is a well-known ornamental plant with ornate flowers blooming in spring in mainland China. Its dried roots have been used in traditional Chinese medicine for a long time, with claims being made concerning its antispasmodic, anti-inflammatory, antitumor, tonic, astringent, and analgesic properties [1][2]. Chemical investigations of this genus afforded monoterpene derivatives, polyphenols, and triterpenoids [3–5]. Several biological effects, such as anticoagulant, anti-inflammatory, anti-allergic, analgesia, and antithrombotic activities, may be attributed to the characteristic chemotaxonomic markers paeoniflorin and its derivatives [6][7]. Previously, we reported the isolation and structural elucidation of monoterpene glycosides and phenolic glycosides from *P. lactiflora* [3][8]. In our continuing search for the bioactive components from the genus *Paeonia*, a re-investigation of the roots of *P. lactiflora* led to the discovery of two new and seven known triterpenoids. Herein, we report the isolation, structure elucidation, and anti-inflammatory and cytotoxic activities of these compounds.

Results and Discussion. – In this article, we report the isolation and structural elucidation of two new pentacyclic triterpenoids, paeonenoides D and E (**1** and **2**, resp.), along with seven known compounds, 11α,12α-epoxy-3β,4β-dihydroxy-24-norolean-28-oic acid (**3**) [5], 3β,4β,23,29-tetrahydroxy-24-norolean-12-en-28-oic acid (**4**) [5], paeonenoide A (**5**) [9], 11α,12α-epoxy-3β,23-dihydroxyolean-28,13β-olide (**6**) [10], 11α,12α-epoxy-3β-hydroxyolean-28,13β-olide (**7**) [11], oleanolic acid (**8**) [10], and hederagenine (**9**) [10], by comparing their NMR data with those reported in the literature.

Compound **1** has the molecular formula $C_{29}H_{46}O_6$, as indicated by the HR-ESI-MS *pseudo*-molecular-ion peak in negative mode at *m*/*z* 489.3221 ($[M-H]^-$, $C_{29}H_{45}O_6^-$; calc. 489.3222), suggesting that **1** is a nortriterpenoid metabolite. This deduction was confirmed by ^{13}C- and DEPT-NMR spectra which exhibited signals for 29 C-atoms. The ^{1}H- and ^{13}C-NMR (*Table 1*), and HMQC spectra of **1** revealed that it contained five Me, nine CH_2, one CH_2O, four CH, and three CH–O groups, six C_q-atoms (one O-bearing), and one COOH group. The ^{1}H-NMR spectrum showed typical resonances for an epoxide ring at δ(H) 3.06, (*dd*, *J* = 6.0, 2.0, 1 H) and 3.11 (*dd*, *J* = 6.0, 2.0, 1 H) in α-orientation at C(11) and C(12). This deduction was confirmed by the HMQCs H–C(11)/C(11) and H–C(12)/C(12) and by the HMBCs H–C(11)/C(9,12) and H–C(12)/C(11,13). In addition, a COOH group could be located at C(17) on the basis of the long-range HMBCs H–C(16,18,22)/C(28). Comparison of the NMR data for **1** with those for the known compound 11α,12α-epoxy-3β,4β-dihydroxy-24-norolean-28-oic acid (**3**) [5] revealed that the two compounds are similar, suggesting that they have the same skeleton. The only difference was the absence of the signal of the Me(23) group which was replaced by a CH_2O group in **1**. The axial orientation of H–C(3) was supported by ROESY signals between H–C(3) and H_α–C(1), revealing that the OH group at C(3) is β-oriented. Consequently, the structure of **1** was determined as shown in *Fig. 1*, and the compound was named paeonenoide D.

The HR-ESI-MS *pseudo*-molecular-ion peak of **2** at *m*/*z* 505.3160 ($[M-H]^-$, $C_{29}H_{45}O_7^-$; calc. 505.3171) supported the molecular formula $C_{29}H_{46}O_7$, suggesting this compound to be a further nortriterpenoid derivative. Comparison of the NMR data for **2** and **1** revealed that the two compounds are similar, except for the absence of the signal due to the Me(29) group in **1**. Instead, the signal of an additional HO–CH_2 group at δ(C) 65.2 (C(29)) was

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Table 1. *^{1}H- and ^{13}C-NMR Data* (500 and 125 MHz, resp.; in C_5D_5N) *for* **1** *and* **2**

Position	**1**		**2**	
	δ(H)	δ(C)	δ(H)	δ(C)
1	1.02 – 1.09 (*m*), 1.62 – 1.68 (*m*)	38.5	1.03 – 1.10 (*m*), 1.62 – 1.68 (*m*)	38.4
2	1.61 – 1.68 (*m*), 1.82 – 1.87 (*m*)	27.2	1.61 – 1.68 (*m*), 1.83 – 1.88 (*m*)	27.2
3	3.42 (*dd*, *J* = 10.0, 4.5)	76.4	3.43 (*dd*, *J* = 10.0, 4.5)	76.4
4		74.2		74.2
5	1.14 (*dd*, *J* = 6.5, 3.5)	47.6	1.14 (*dd*, *J* = 6.5, 3.5)	47.5
6	1.30 – 1.38 (*m*), 1.52 – 1.59 (*m*)	18.2	1.31 – 1.38 (*m*), 1.53 – 1.60 (*m*)	18.2
7	1.42 – 1.48 (*m*), 1.60 – 1.67 (*m*)	31.9	1.42 – 1.48 (*m*), 1.61 – 1.68 (*m*)	32.0
8		42.2		42.2
9	1.62 (*d*, *J* = 6.0)	50.6	1.63 (*d*, *J* = 6.0)	50.7
10		36.6		36.5
11	3.11 (*dd*, *J* = 6.0, 2.0)	53.1	3.12 (*dd*, *J* = 6.0, 2.0)	53.1
12	3.06 (*dd*, *J* = 6.0, 2.0)	56.7	3.08 (*dd*, *J* = 6.0, 2.0)	56.7
13	1.22 – 1.28 (*m*)	37.2	1.22 – 1.28 (*m*)	37.2
14		41.2		41.2
15	1.11 – 1.17 (*m*), 1.82 – 1.89 (*m*)	26.2	1.12 – 1.18 (*m*), 1.82 – 1.89 (*m*)	26.2
16	1.22 – 1.28 (*m*), 1.74 – 1.81 (*m*)	23.3	1.23 – 1.29 (*m*), 1.74 – 1.81 (*m*)	23.2
17		45.6		45.6
18	2.22 – 2.28 (*m*)	33.6	2.23 – 2.29 (*m*)	33.8
19	1.12 – 1.18 (*m*)	41.5	1.13 – 1.19 (*m*)	42.4
	1.66 – 1.71 (*m*)		1.68 – 1.74 (*m*)	
20		31.6		30.6
21	1.16 – 1.24 (*m*), 1.39 – 1.46 (*m*)	34.3	1.18 – 1.26 (*m*), 1.42 – 1.49 (*m*)	35.4
22	1.43 – 1.50 (*m*), 1.78 – 1.85 (*m*)	33.9	1.54 – 1.61 (*m*), 1.82 – 1.89 (*m*)	34.3
23	3.41 (*d*, *J* = 10.5), 3.62 (*d*, *J* = 10.5)	64.1	3.42 (*d*, *J* = 10.5), 3.63 (*d*, *J* = 10.5)	64.1
25	1.10 (*s*)	17.2	1.11 (*s*)	17.2
26	1.19 (*s*)	18.5	1.20 (*s*)	18.5
27	1.10 (*s*)	19.8	1.11 (*s*)	19.7
28		180.2		180.1
29	1.09 (*s*)	32.7	3.41 (*d*, *J* = 10.0), 3.61 (*d*, *J* = 10.0)	65.2
30	1.06 (*s*)	24.9	1.02 (*s*)	23.9

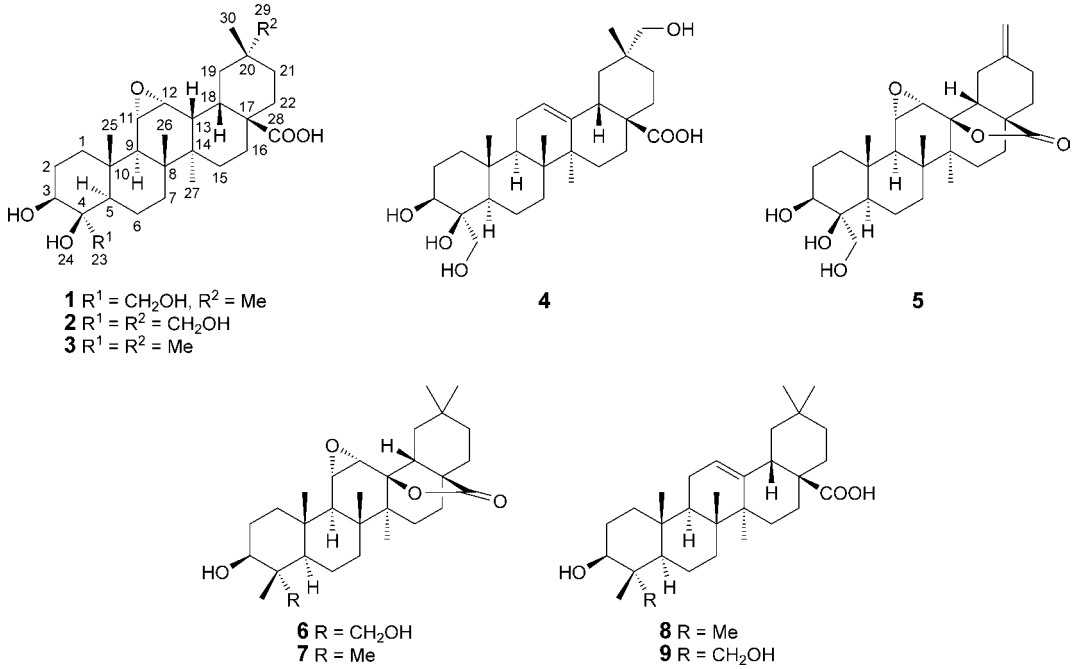

Fig. 1. *Structures of* **1** – **9**

present in **2**, which was confirmed by the HMBC H–C(29)/C(21). Thus, the structure of **2** was determined as shown in *Fig. 1*, and the compound was named paeonenoide E. The HMBCs of **1** and **2** are illustrated in *Fig. 2*.

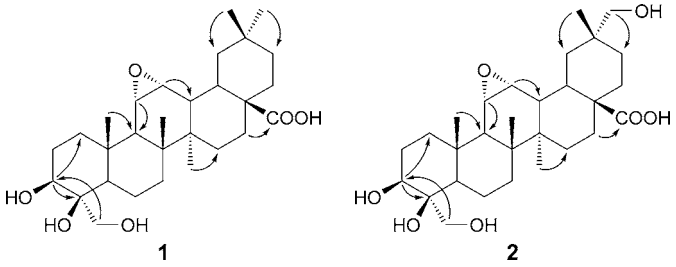

Fig. 2. *Key HMBCs* (H → C) *of* **1** *and* **2**

Effect of the Inhibitory Activity against Lipopolysaccharide (LPS)-Induced NO Production. Compounds **1**–**7** were screened for inhibitory activities against LPS-induced NO production, according to the methods described by *Islam et al.* [12]. Dexamethasone (*Sigma*, St. Louis, MO, USA) was used as the positive control with an IC_{50} value of 0.7 ± 0.08 μM ($n = 3$). Compounds **1**–**3** and **5**–**7** exhibited inhibitory activities with IC_{50} values of 9.6 ± 1.6, 10.2 ± 2.2, 12.2 ± 1.9, 22.9 ± 3.8, 27.3 ± 3.1, and 32.2 ± 3.7 μM ($n = 3$), respectively (*Table 2*). Unexpectedly, **4** did not show any inhibitory activity. The triterpenoids with an epoxide ring and a free COOH function, **1**–**3**, showed significantly increased activities compared with the other pentacyclic triterpenoids. No cytotoxic activity was observed for **1**–**7** in correspondingly treated cells (cell viability > 90%).

Table 2. IC_{50} *Values of* **1**–**7** *for NO Production in LPS-Stimulated RAW264.7 Cells*

Compound	IC_{50} [μM]
1	9.6 ± 1.6
2	10.2 ± 2.2
3	12.2 ± 1.9
4	> 100
5	22.9 ± 3.8
6	27.3 ± 3.1
7	32.2 ± 3.7
Dexamethasone[a])	0.7 ± 0.08

[a]) Positive control.

Cytotoxic Activity. Compounds **1**–**7** were evaluated for their cytotoxic activities against human leukemia (HL-60), human hepatocellular carcinoma (Hep-G2), and human ovarian (SK-OV-3) cell lines. Doxorubicin was used as positive control, and the results are shown in *Table 3*. Among the seven compounds tested in the present experiment, six triterpenoids, **1**–**6**, showed significant cytotoxic activities against the Hep-G2 cell line with IC_{50} values in the range of 12.2 – 18.3 μM. Compounds **3** and **4** showed moderate cytotoxic activities against the HL-60 cell line, as previously reported [5]. Furthermore, all compounds exhibited modest cytotoxic activities against the HL-60 and SK-OV-3 cell lines with IC_{50} values in the range of 19.2 – 51.1 μM.

Table 3. *Inhibitory Effects of* **1**–**7** *against the Three Cancer Cell Lines HL-60, Hep-G2, and SK-OV-3*

Compound	IC_{50} [μM]		
	HL-60	Hep-G2	SK-OV-3
1	35.2 ± 2.6	18.3 ± 1.2	19.2 ± 1.5
2	42.6 ± 3.3	17.2 ± 1.1	23.9 ± 0.8
3	29.3 ± 1.9	14.2 ± 0.9	27.5 ± 2.1
4	32.7 ± 2.7	12.2 ± 0.6	29.0 ± 1.8
5	27.7 ± 2.0	15.1 ± 1.3	27.3 ± 0.8
6	24.4 ± 3.1	13.2 ± 1.9	22.0 ± 1.4
7	51.1 ± 4.2	23.9 ± 2.1	31.0 ± 2.5
Mitoxantrone[a])	8.2 ± 0.7	10.3 ± 0.9	6.9 ± 0.5

[a]) Positive control.

Experimental Part

General. Column chromatography (CC): silica gel (SiO_2; *Merck*, Darmstadt, Germany), reversed-phase C_{18} SiO_2 (*RP-C_{18}*; *Merck*), and *Sephadex LH-20* (*GE Healthcare*, MA, USA). HPLC: *ODS* column (*YMC-pack ODS-A*; 250 × 10 mm i.d., 5 μm; *YMC*, Kyoto, Japan) with an *Alltech* evaporative light scattering detector. Optical rotations: *JASCO P-1020* digital polarimeter (*Jasco*, Tokyo, Japan). IR Spectra: *Bruker IFS-55 plus* spectrometer (*Bruker*, Ettlingen, Germany); KBr; $\tilde{\nu}$ in cm^{-1}. ^{1}H-, ^{13}C-, and 2D-NMR spectra: *Inova 500* spectrometer (500 and 125 MHz for ^{1}H and ^{13}C, resp.; *Bruker*, Waltham, MA, USA); in C_5D_5N; δ in ppm rel. to Me_4Si as internal standard, *J* in Hz. ESI-MS: *Agilent 6320* ion trap mass spectrometer (*Agilent*, Santa Clara, CA, USA); in *m*/*z*. HR-ESI-MS: *Bruker-Daltonics APEX-III 7.0 TESLA* FTMS spectrometer (*Bruker*, Billerica, MA, USA); in *m*/*z*.

Plant Material. Roots of *P. lactiflora* were collected in May 2014 in Chengdu, Sichuan Province, P. R. China. The identification of the plant was performed by *Q. F.* A voucher specimen (PL 201403) is maintained with the Herbarium of the Faculty of Biotechnology Industry, Chengdu University.

Extraction and Isolation. Dried roots of *P. lactiflora* (5.8 kg) were extracted with EtOH. After removing the solvent, the residue (735 g) was suspended in H_2O and extracted with $CHCl_3$ (57 g). The $CHCl_3$ extract was subjected to CC (SiO_2; petroleum ether/AcOEt 10 : 1 – 2 : 1 and $CHCl_3$/MeOH 10 : 1 – 3 : 1) to afford six fractions, *Frs. 1* (8.1 g), *2* (6.9 g), *3* (13.2 g), *4* (3.5 g), *5* (9.3 g), and *6* (7.5 g). *Fr. 2* was subjected to CC (*RP-C_{18}*; MeOH/H_2O 80 : 20) to give three subfractions, *Frs. 2.1 – 2.3*. *Fr. 2.2* (219 mg) was separated by prep. HPLC (MeCN/H_2O 63 : 37) to give **5** (15.2 mg), **6** (12.3 mg), and **7** (21.6 mg). *Fr. 3* was subjected to CC (*RP-C_{18}*; MeOH/H_2O 70 : 30 – 85 : 15, in gradient) to give four subfractions, *Frs. 3.1 – 3.4*. *Fr. 3.1* (108 mg) was resubjected to CC (*Sephadex LH-20*; $CHCl_3$/MeOH 30 : 70) followed by prep. HPLC to obtain **1** (12.5 mg) and **3** (13.7 mg). *Fr. 3.2* (77 mg) was purified by prep. HPLC (MeOH/H_2O 73 : 27) to afford **2** (15.9 mg). *Fr. 3.3* (91 mg) was subjected to prep. HPLC (MeOH/H_2O 73 : 27) to afford **4** (19.3 mg), **8** (18.2 mg), and **9** (29.3 mg).

Paeonenoide D (=*11α,12α-Epoxy-3β,4β,23-trihydroxy-24-norolean-28-oic Acid*; **1**). Colorless powder. $[\alpha]_D^{25} = +31.4$ ($c = 0.2$, MeOH). IR: 3420, 2790, 1642, 1330, 1010. ^{1}H- and ^{13}C-NMR data: *Table 1*. HR-ESI-MS: 489.3221 ($[M-H]^-$, $C_{29}H_{45}O_6^-$; calc. 489.3222).

Paeonenoide E (=*11α,12α-Epoxy-3β,4β,23,29-tetrahydroxy-24-norolean-28-oic Acid*; **2**). Colorless powder. $[\alpha]_D^{25} = +32.6$ ($c = 0.2$, MeOH). IR: 3422, 2791, 1642, 1330, 1010. ^{1}H- and ^{13}C-NMR data: *Table 1*. HR-ESI-MS: 505.3160 ($[M-H]^-$, $C_{29}H_{45}O_7^-$; calc. 505.3171).

Assay for Inhibitory Activity against LPS-Induced NO Production. The anti-inflammatory activities of the compounds were evaluated by determining the amount of NO_2^-, a stable oxidized product in cell culture supernatant, as described previously [12]. Briefly, cells (1×10^5

cells/well) were cultured in 48-well plates overnight and replaced by the media containing 1 μg ml^{-1} of LPS (*Sigma*, St. Louis, MO, USA) and different concentrations of the compounds. After culturing for 48 h, 50 μl of the supernatant were removed and mixed with an equal volume of *Griess* reagent (*Sigma*, St. Louis, MO, USA) for 15 min. The absorbance at 540 nm was measured with a microplate reader (*Spectra Classic*; *Tecan*, Salzburg, Austria). Concentrations of NO_2^- in the supernatant were determined by comparison with a $NaNO_2$ standard curve. Cell viability was measured with the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2*H*-tetrazolium bromide (MTT)-based colorimetric assay. Experiments were performed in triplicate and mean activity ± SD calculated.

Assay for Cytotoxic Activity. Cells were seeded at the density 2×10^4 cells/well in a 96-well plate and pre-incubated for 24 h. Test samples were dissolved in a small amount of DMSO and diluted in the appropriate culture medium (final concentration of DMSO $< 0.5\%$). After removal of pre-incubated culture medium, medium (100 μl) containing various concentrations of test compounds was added and the cells were further incubated for 48 h. Cell viability was determined by MTT colorimetric assay [13]. Absorbance values at 550 nm were measured with a microplate reader. The cytotoxic activity was expressed as an IC_{50} value which is the concentration to reduce the absorbance of treated cells by 50% with reference to the control. Experiments were performed in triplicate and mean activity ± SD calculated.

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