Two New Megastigmane Glycosides, Physanosides A and B, from *Physalis alkekengi* L. var. *franchetii*, and Their Effect on NO Release in Macrophages

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Two new megastigmane glycosides, physanosides A and B (**1** and **2**, resp.), were isolated from *Physalis alkekengi* L. var. *franchetii*, together with four known compounds (6*S*,9*R*)-roseoside (**3**), (6*S*,9*S*)-roseoside (**4**), (6*R*,9*S*)-3-oxo-α-ionol β-D-glucopyranoside (**5**), and citroside A (**6**). Their structures were elucidated on the basis of physicochemical evidence, in-depth NMR spectroscopic analysis, high-resolution mass spectrometry, and CD spectroscopy, and their inhibitory effect on NO production was also examined. Compounds **2** and **3** exhibited strong inhibition on lipopolysaccharide-induced NO release by macrophages with IC_{50} values of 9.93 and 7.31 μM, respectively.

Introduction. – The plant *Physalis alkekengi* L.var. *franchetii* (Solanaceae) is a perennial herb widely distributed on the mountain slopes in Northeast of China and is a traditional Chinese herb medicine for the treatment of sore throat, cough, eczema, hepatitis, urinary problems, and tumors [1]. In previous studies, physalins [2–10], neophysalins [11][12], alkaloids [13][14], and flavonoids [15] have been isolated from this herb medicine. During the course of our studies on bioactive constituents of *Physalis alkekengi* L. var. *franchetii*, two new megastigmane glycosides, **1** and **2**, were isolated along with four known ones **3**–**6**. This paper describes the isolation and structural elucidation of these compounds and their inhibitory effects on NO production in mouse monocyte macrophages.

Results and Discussion. – Physanoside A (**1**) was obtained as a white, amorphous powder. Its molecular formula was determined to be $C_{25}H_{40}O_{12}$ by HR-ESI-MS (*m*/*z* 555.2414 $[M+Na]^+$, $C_{25}H_{40}NaO_{12}^+$; calc. 555.2417). The UV spectrum of **1** (λ_{max} 237 nm) indicated the presence of an α,β-unsaturated ketone. The ^{1}H-NMR spectrum of **1** showed signals of a vinyl H-atom at δ 5.81 (*s*, H–C(4)), of two mutually coupled vinyl H-atoms at δ 5.79 (*dd*, *J* = 9.4, 15.2, H–C(7)) and 5.49 (*dd*, *J* = 6.5, 15.2, H–C(8)), of two CH groups at δ 4.36–4.38 (*m*, H–C(9)) and 2.61 (*d*, *J* = 9.4, H–C(6)), of one CH_2 group at δ 2.41 (*d*, *J* = 16.4, 1 H–C(2)) and 1.95 (*d*, *J* = 16.4, 1 H–C(2)), of a

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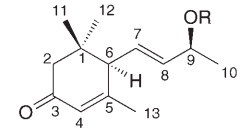
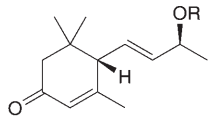
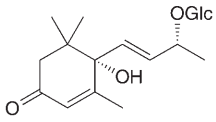


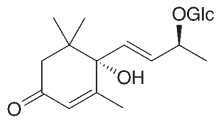
1 (6*R*,9*S*) R = Glc(1→6)Glc
5 (6*R*,9*S*) R = Glc



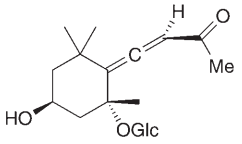
2 (6*S*,9*S*) R = Glc(1→6)Glc



3 (6*S*,9*R*)



4 (6*S*,9*S*)



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secondary Me group (δ 1.19 (*d*, $J = 6.4$, Me(10)) and of three tertiary Me groups (δ 1.88, 0.95, 0.91 (each *s*, Me(13), Me(11), Me(12), resp.). Two characteristic anomeric H-atom signals at δ 4.10 (*d*, $J = 7.8$, 1 H) and 4.29 (*d*, $J = 7.8$, 1 H) suggested the presence of two sugar residues in **1**. Acid hydrolysis of **1** produced glucose as sugar residues [16], and the *J* values of the anomeric H-atom signals indicated the β-orientation of the glucosidic linkages. Moreover, enzymatic hydrolysis of **1** by β-glucosidase (emulsin) yielded the aglycone, which indicate the β-D-configuration of the glucose residues [17]. The ^{13}C-NMR spectrum of **1** displayed 25 C-atom signals, among which 13 signals were similar to those corresponding to the aglycone moiety of 3-oxo-α-ionol glucoside [18]. The remaining twelve C-atom resonances could be superimposed on signals due to two β-D-glucopyranosyl units (*Table 1*). The location of the sugar residues in **1** was established by the HMBC experiment. The anomeric H-atom signal (δ 4.10 (*d*, $J = 7.8$, H−C(1′))), which was assigned from the TOCSY spectrum, was correlated through a three-bond coupling with C(9) (δ 72.5) of the aglycone, and the H-atom signal at δ 4.36–4.38 (*m*, H−C(9)) was correlated, in turn, to the anomeric C-atom signal at δ 100.3 (C(1′)). The other anomeric H-atom signal at δ 4.29 (*d*, $J = 7.8$, H−C(1″)) was correlated with C(6′) (δ 68.1) of glucose-1, and the H-atom signals at δ 3.94 (br. *d*, $J =$ 10.7, H−C(6′)) and 3.57 (*dd*, $J = 6.8$, 10.7, H−C(6′)) were correlated with the anomeric C-atom signal at δ 103.4 (C(1″)) of glucose-2. This indicated that a glucopyranosyl-(1″→6′)-glucopyranosyl disaccharide moiety was located at C(9) of a 3-oxo-α-ionyl moiety. The CD spectrum of **1** showed a $\Delta\varepsilon$ value of +109.3 mdeg at 242.5 nm, which was similar to that of eriojaposide A [19] and (6*R*,9*S*)-3-oxo-α-ionol β-D-glucopyranoside [18], indicating the (6*R*)-configuration. The absolute configuration at C(9) of the aglycone was assigned as (*S*) on the basis of the diagnostic chemical shift of the C(9) signal (δ 72.5) in the ^{13}C-NMR spectrum [17–20]. Consequently, the structure of **1** was determined to be (6*R*,9*S*)-3-oxo-α-ionyl-9-*O*-β-D-glucopyranosyl-(1″→6′)-β-D-glucopyranoside.

Physanoside B (**2**) was obtained as a white, amorphous powder. Its molecular formula was determined to be $C_{25}H_{40}O_{12}$ by HR-ESI-MS (*m*/*z* 555.2419 $[M+Na]^+$, $C_{25}H_{40}NaO_{12}^+$; calc. 555.2417). The H- and C-atom signals in the NMR spectra of **2** (*Table 1*) were very similar to those of **1**. The HSQC, HMBC, NOESY, and TOCSY

Table 1. *NMR Data of* **1** *and* **2**. At 600 (^{1}H) and 150 MHz (^{13}C); in (D_6)DMSO; δ in ppm, J in Hz.

Position	**1**		**2**	
	δ(C)	δ(H)	δ(C)	δ(H)
1	35.8	–	35.8	–
2	47.3	2.41 (*d*, *J*=16.4), 1.95 (*d*, *J*=16.4)	47.5	2.37 (*J*=16.4), 1.97 (*d*, *J*=16.4)
3	198.1	–	198.1	–
4	125.0	5.81 (*s*)	125.1	5.80 (*s*)
5	162.0	–	161.8	–
6	54.8	2.61 (*d*, *J*=9.4)	54.8	2.64 (*d*, *J*=9.4)
7	128.9	5.79 (*dd*, *J*=15.2, 9.4)	129.5	5.72 (*dd*, *J*=15.2, 9.4)
8	135.1	5.49 (*dd*, *J*=15.2, 6.5)	135.4	5.47 (*dd*, *J*=15.2, 7.4)
9	72.5	4.36–4.38 (*m*)	72.6	4.36–4.38 (*m*)
10	22.1	1.19 (*d*, *J*=6.4)	22.0	1.19 (*d*, *J*=6.4)
11	27.5	0.95 (*s*)	27.6	0.96 (*s*)
12	26.9	0.91 (*s*)	26.7	0.92 (*s*)
13	23.0	1.88 (*s*)	23.0	1.86 (*s*)
Glc-1				
1′	100.3	4.10 (*d*, *J*=7.8)	99.9	4.14 (*d*, *J*=7.8)
2′	73.2	2.93–2.98 (*m*)	73.2	2.93–2.98 (*m*)
3′	76.2	3.18–3.21 (*m*)	76.3	3.20–3.23 (*m*)
4′	70.0	3.04–3.08 (*m*)	70.1	3.04–3.08 (*m*)
5′	76.9	3.04–3.08 (*m*)	76.9	3.04–3.08 (*m*)
6′	68.1	3.94 (*d*, *J*=10.7) 3.57 (*dd*, *J*=10.7, 6.8)	68.1	3.95 (br. *d*, *J*=11.7) 3.58 (*dd*, *J*=11.7, 6.8)
Glc-2				
1″	103.4	4.29 (*d*, *J*=7.8)	103.4	4.30 (*d*, *J*=7.8)
2″	73.6	2.93–2.98 (*m*)	73.6	2.93–2.98 (*m*)
3″	76.9	3.04–3.08 (*m*)	76.7	3.04–3.08 (*m*)
4″	70.1	3.04–3.08 (*m*)	70.2	3.04–3.08 (*m*)
5″	77.0	3.09–3.11 (*m*)	77.0	3.09–3.11 (*m*)
6″	61.1	3.66 (br. *d*, *J*=11.5), 3.43 (*dd*, *J*=11.5, 5.7)	61.2	3.67 (br. *d*, *J*=11.4), 3.43 (*dd*, *J*=11.4, 6.8)

spectra revealed that **2** had the same planar structure as **1**. The ^{13}C-NMR of **2** showed an oxygenated CH C-atom resonance at δ 72.6 for C(9), which was nearly the same as that for **1** (δ 72.5, C(9)), indicating the same absolute configuration at C(9) of **2** as that of **1** [17][18]. In contrast to **1**, the CD spectrum of **2** showed a $\Delta\varepsilon$ value of -42.1 mdeg at 242.5 nm indicating the (*S*)-configuration at C(6) [18]. Hence, physanoside B (**2**) was the 6-epimer of physanoside A (**1**); consequently, the structure of **2** was determined to be (6*S*,9*S*)-3-oxo-α-ionyl-9-*O*-β-D-glucopyranosyl-(1″→6′)-β-D-glucopyranoside.

In addition to the new megastigmane glucosides **1** and **2**, four known compounds, (6*S*,9*R*)-roseoside (**3**) [17][21], (6*S*,9*S*)-roseoside (**4**) [17], (6*R*,9*S*)-3-oxo-α-ionol β-D-glucopyranoside (**5**) [19], and citroside A (**6**) [22], were also isolated and identified by comparison of their spectral data with those reported in the literature. Compounds **3**–**6** are found for the first time in *Physalis alkekengi* L. var. *franchetii*.

Compounds **1**–**6** were examined for their inhibitory effects on NO production induced by lipopolysaccharide (LPS) in macrophages. Cell viability was checked by the

MTT method. Compounds **1**–**6** exhibited no cytotoxicity during the experiment (data not shown). The IC_{50} values are shown in *Table 2*. Compounds **2** and **3** showed strong inhibition on NO production induced by LPS in macrophages with IC_{50} values of 9.93 and 7.31 μM, respectively.

Table 2. *Effect of Compounds* **1**–**6** *on the NO Production Induced by LPS in Macrophages*

Compound	$IC_{50} \pm$ S.D. [μM]
1	> 100
2	9.93 ± 1.52
3	7.31 ± 1.18
4	> 100
5	> 100
6	> 100
Hydrocortisone	64.34 ± 7.49

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Experimental Part

General. Prep. HPLC: *Phenomsil Prep-ODS C18* column (10 mm i.d. × 250 mm, 10 μm); flow rate of 3 ml/min, UV detection at 235 nm, HPLC-grade MeOH and double-distilled H_2O as solvents. TLC: Silica gel GF_{254} (*Qingdao Haiyang*, *Co.*, Qingdao, China). Column chromatography (CC): Polyamide (80–140 mesh; *Taizhou Luqiao Sijia Biochemical Plastics Company*, Zhejiang, China), *Sephadex LH-20* (*Pharmacia Biotech*, Sweden), *RP-18* (40–75 mm, *Germany Merk Chemical Ltd.*). Optical rotations: *Perkin-Elmer 241* polarimeter. UV Spectra: *Shimadzu UV-2201* spectrophotometer; λ in nm (log ε). CD Spectra: *Jasco P-1020* digital spectrometer; λ in nm ($\Delta\varepsilon$ in mdeg). IR Spectra: *Bruker IFS-55* spectrometer; in cm^{-1}. NMR Spectra: *Bruker ARX-300* and *ARX-600* apparatus, in (D_6)DMSO; δ in ppm rel. to Me_4Si, *J* in Hz. ESI-MS: *Agilent 1100-LC/MSDTrapSL* mass spectrometer; in *m*/*z*. HR-ESI-MS: *Agilent 6210-TOF* mass spectrometer; in *m*/*z*.

Plant Material. The dried leaves and stems of *Physalis alkekengi* L. var. *franchetii* were collected in September 2004, from the locality Yilan, Heilongjiang Province, China. It was authenticated by Prof. *Qi-Shi Sun*, Department of Pharmaceutical Botany, School of Traditional Chinese Materia Medica, Shenyang Pharmaceutical University. A voucher specimen (collection number: 20041018) was deposited with the herbarium of the laboratory of Natural Products Chemistry, Shenyang Pharmaceutical University.

Extraction and Isolation. The dried leaves and stems (7 kg) of *P. alkekengi* L. var. *franchetii* were extracted with 60% aq. EtOH (20 l, 3 × 2 h) under reflux. The resulting EtOH extract was concentrated *in vacuo*, suspended in H_2O (2000 ml), and partitioned successively with cyclohexane (3 × 2000 ml), AcOEt (3 × 2000 ml), and BuOH (3 × 2000 ml). The BuOH fraction (100 g) was subjected to CC (polyamide; gradient H_2O/MeOH 100 : 0, 90 : 10, 70 : 30, 50 : 50, 30 : 70, 0 : 100) to yield fractions *Fr. B1–B6*. *Fr. B1* (7.8 g) was subjected to CC (*Sephadex LH-20*; MeOH/H_2O 1 : 1) to yield *Fr. B11–B13*. *Fr. B12* (3.2 g) was subjected to CC (*RP-18*; gradient H_2O/MeOH 100 : 0, 90 : 10, 70 : 30, 50 : 50, 30 : 70, 0 : 100) to yield *Fr. B121–B126*. *Fr. B122* (320 mg) was purified on prep. HPLC to afford compound **1** (18 mg, H_2O/MeOH 80 : 20, t_R 32.7 min) and **2** (9 mg, H_2O/MeOH 70 : 30, t_R 31.1 min). *Fr. B123* (500 mg) was purified on prep. HPLC to provide compound **3** (12.5 mg, H_2O/MeOH 70 : 30, t_R 23.8 min), **4** (50 mg, H_2O/MeOH 70 : 30, t_R 25.4 min), **5** (37 mg, H_2O/MeOH 70 : 30, t_R 30.4 min), and **6** (50 mg, H_2O/MeOH 70 : 30, t_R 19.4 min).

Determination of NO Inhibition. Mouse monocyte-macrophages RAW 264.7 (ATCC TIB-71) were purchased from the Chinese Academy of Science. RPMI 1640 medium, penicillin, streptomycin, and fetal

bovine serum were purchased from *Invitrogen* (N.Y., USA). Lipopolysaccharide (LPS), DMSO, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2*H*-tetrazolium bromide (MTT), and hydrocortisone were obtained from *Sigma Co.* RAW 264.7 Cells were maintained in RPMI 1640 medium supplemented with penicillin (100 U/ml), streptomycin (100 μg/ml), and 10% heat-inactivated fetal bovine serum at 37° in a humidified incubator with 5% CO_2 and 95% air. The medium was routinely changed every two days. RAW 264.7 Cells were passaged by trypsinization until they attained confluence and were used for assays during the exponential growth phase.

Compounds **1**–**6** were dissolved in cell culture level DMSO and were further diluted with the culture medium to give a final DMSO concentration of 0.2% in assay. This concentration of DMSO had no significant effect on the growth of the cell line tested. Cell concentration was adjusted to $5 \cdot 10^5$ cells/ml, and 200 μl were seeded in every well of a 96-well plate. After 1 h incubation, the cells were treated with 1 μg/ml of LPS and various concentrations of test compounds for 24 h. Control groups received an equal amount of DMSO. As a parameter of NO release, the nitrite concentration was measured in the supernatant of RAW 264.7 cells by the *Griess* reaction. Briefly, 100 μl of culture medium in each well were taken out to another plate, and the level of NO were assessed by measuring the accumulation of nitrite (NO_2^-) using 100 μl of *Griess* agent (mixture of 0.1% *N*-[naphthalen-1-yl]ethylenediamine in 5% phosphoric acid and 1% sulfanilamide). The concentration of NO_2^- was calculated by a working line from 0, 1, 2, 5, 10, 20, 50, 100 μM $NaNO_2$ solns. The inhibitory rate on NO production induced by LPS was calculated by the NO_2^- levels as follows:

$$\text{Inhibitory rate } [\%] = 100 \times \frac{[NO_2^-]_{LPS} - [NO_2^-]_{LPS+sample}}{[NO_2^-]_{LPS} - [NO_2^-]_{untreated}}$$

Every experiment was performed in triplicate; data are expressed as mean ± S.D. of three independent experiments.

Physanoside A (=*(6*R,*9*S*)-3-Oxo-α-ionyl-9*-O-*β*-D-*glucopyranosyl-(1″→6′)-β*-D-*glucopyranoside*=*(2*S,*3*E*)-4-[(1*R*)-2,6,6-Trimethyl-4-oxocyclohex-2-en-1-yl]but-3-en-2-yl 6*-O-*β*-D-*Glucopyranosyl-β*-D-*glucopyranoside*; **1**). White, amorphous powder. $C_{25}H_{40}O_{12}$. $[\alpha]_D^{20} = +41.2$ ($c = 0.13$, MeOH). UV (MeOH): 237 (4.07). CD ($c = 0.28\ g \cdot l^{-1}$, MeOH): 242.5 (+109.3). IR (KBr): 3384, 2930, 1655, 1027, 613. ^{1}H- (600 MHz) and ^{13}C-NMR (150 MHz): see *Table 1*. ESI-MS (pos./neg.): 555 ($[M+Na]^+$), 567.5 ($[M+Cl]^-$), 531($[M-H]^-$). HR-ESI-MS: 555.2414 ($[M+Na]^+$, $C_{25}H_{40}NaO_{12}^+$; calc. 555.2417).

Physanoside B (=*(6*S,*9*S*)-3-Oxo-α-ionyl-9*-O-*β*-D-*glucopyranosyl-(1″→6′)-β*-D-*glucopyranoside*=*(2*S,*3*E*)-4-[(1*S*)-2,6,6-Trimethyl-4-oxocyclohex-2-en-1-yl]but-3-en-2-yl 6*-O-*β*-D-*Glucopyranosyl-β*-D-*glucopyranoside*; **2**). White, amorphous powder. $C_{25}H_{40}O_{12}$. $[\alpha]_D^{20} = -106.1$ ($c = 0.09$, MeOH). UV (MeOH): 236 (3.88). CD ($c = 0.24\ g \cdot l^{-1}$; MeOH): 242.5 (−42.1). IR (KBr): 3422, 2925, 1650, 1073, 602. ^{1}H- (600 MHz) and ^{13}C-NMR (150 MHz): see *Table 1*. HR-ESI-MS: 555.2419 ($[M+Na]^+$, $C_{25}H_{40}NaO_{12}^+$; calc. 555.2417).

Acid Hydrolysis of **1** *and* **2**. Each compound (1.5 mg) was heated in an ampoule with 1.5 ml of aq. 15% HCl at 110° for 2 h. The aglycone was extracted with CH_2Cl_2 three times, and the aq. residue was evaporated under reduced pressure. Then, 1 ml of pyridine and 2 mg of $NH_2OH \cdot HCl$ were added to the residue, and the mixture was heated at 100° for 1 h. After cooling, Ac_2O (0.5 ml) was added, and the mixture was heated at 100° for 1 h. The mixtures were evaporated under reduced pressure, and the resulting aldononitrile peracetates were analyzed by GC/MS using standard aldononitrile peracetates as reference samples.

Enzymatic Hydrolysis of **1** *and* **2**. The solns. of **1** and **2** (each 2 mg) in acetate buffer (pH 4.4, 2 ml) were treated each with 10 mg of β-glucosidase (emulsin (EC 3.2.1.21, *Sigma*)), and the solns. were incubated at 37° for 48 h. The solns. were extracted with the same volume of AcOEt. The aglycone was analyzed by ESI-MS spectroscopy.

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