Steroids and Flavonoids from *Physalis alkekengi* var. *franchetii* and Their Inhibitory Effects on Nitric Oxide Production

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Four new steroidal compounds possessing a rare 13,14-seco-16,24-cycloergostane skeleton, physalin Y (**1**), physalin Z (**2**), physalin I (**6**), and physalin II (**7**), were isolated from the 80% EtOH extract of calyces from *Physalis alkekengi* var. *franchetii* together with 11 known steroids (**3**–**5** and **8**–**15**) and eight flavonoids (**16**–**23**). The structures of the new compounds were elucidated primarily on the basis of 1D and 2D NMR and mass spectroscopic studies. The relative configuration of the 3-OH group of **3**, **4**, and **5** was also established or re-established. The inhibitory effects of compounds **1**–**23** on nitric oxide production in lipopolysaccaride-activated macrophages were evaluated.

The calyces of *Physalis alkekengi* var. *franchetii* (Solanaceae) (Chinese name: Jindenglong) are used as a traditional Chinese herbal medicine for the treatment of sore throat, cough, eczema, hepatitis, urinary problems, and tumors.[1] Physalins are the major steroidal constituents of *P. alkekengi* var. *franchetii*, and their rare 13,14-seco-16,24-cycloergostane skeletons have been established by X-ray crystallographic analysis.[2–4] The physalins have been studied extensively during the past 20 years and are reported to have antimycobacterial,[5] immunomodulatory,[6] antitumor,[7] and anti-inflammatory activities.[8] Phytochemical studies on the aerial parts of *Physalis* plants have led to the identification of about thirty physalins.[9–13] The unusual steroids and variety of biological activities of this plant prompted us to extend our studies to the calyces of *P. alkekengi* var. *franchetii*, which resulted in the isolation of 15 physalins (**1**–**15**) and eight flavonoids (**16**–**23**). This paper describes the structural elucidation of four new physalins (**1**, **2**, **6**, **7**) and the inhibitory effect of the isolated compounds on NO production in LPS-activated macrophages.

Results and Discussion

Compound **1** was obtained as a white powder. The molecular formula of **1** was determined to be $C_{28}H_{32}O_{10}$ on the basis of HRESIMS and NMR data (see Experimental Section, Tables 1 and 2). The NMR spectra of **1** were similar to those of physalin B,[14] suggesting that **1** was also a physalin. The ^{1}H NMR spectrum of **1** (in C_5D_5N) showed three methyl singlets at δ 1.19 (H-19), 1.31 (H-28), and 2.37 (H-21), two oxygenated methylene signals at δ 4.06 (1H, m, H-3) and 4.75 (1H, m, H-22), one olefinic proton signal at δ 5.53 (1H, br s, H-6), and two geminally coupled signals of an oxygenated methine at δ 3.99 (1H, d, J = 13.4 Hz, H-27) and 4.79 (1H, dd, J = 13.4, 4.5 Hz, H-27). The HMBC correlations from δ_H 3.99 (H-27), 4.79 (H-27) to δ_C 107.8 (C-14), 31.5 (C-24), 51.0 (C-25) and 167.5 (C-26) and from δ_H 2.96 (H-25) to δ_C 61.6 (C-27) corroborated the existence of a C(14)−O−C(27) ether bridge in **1**. The UV spectrum of **1** showed an absorption maximum at 193 nm (log ϵ 4.1) indicating the absence of a conjugated system in the A/B rings of **1**. The HMBC correlations from δ_H 5.53 (H-6) to δ_C 41.8 (C-4), 56.0 (C-10), 25.9 (C-7), and 40.3 (C-8) confirmed a double bond at C-5 (δ_C 136.2) and C-6 (δ_C 124.2), and the HMBC correlations from δ_H 2.89 (H-2) to δ_C 211.3 (C-1), from δ_H 4.06 (H-3) to δ_C 211.3 (C-1), and from δ_H 2.66 (H-4) to δ_C 49.1 (C-2), 68.8 (C-3), 136.2 (C-5), and 124.2 (C-6) suggested that an OH group was present at C-3. Severe overlapping of the signals of H-2α and H-2β in the ^{1}H NMR spectrum (in C_5D_5N) of **1** did not allow determination of the configuration at C-3; thus, the ^{1}H, ^{13}C, HSQC, HMBC, and NOESY spectra of **1** were remeasured in $CDCl_3$. The NOESY spectrum of **1** (in $CDCl_3$) exhibited NOE correlations between δ_H 1.28 (H-19) and both δ_H 2.83 (H-2β) and 3.77 (H-3) and between δ_H 3.77 (H-3) and δ_H 2.83 (H-2β), indicating β-orientation of H-3 and α-orientation of the 3-OH group. On the basis of the ^{1}H, ^{13}C, and 2D NMR (HSQC, HMBC, NOESY) data, the structure of **1** was unambiguously established as 3α-hydroxy-2,3-dihydrophysalin B, and it was named physalin Y.

Compound **2** was obtained as a white powder with the molecular formula $C_{28}H_{30}O_{10}$, established by the HRESIMS in combination with the NMR data. The NMR data (in DMSO-d_6) of **2** were very similar to those of physalin B,[14] and the differences between them were observed only in their A/B rings. The NMR data of **2** indicated a conjugated diene system composed of a disubstituted double bond (δ_H 5.95 and 6.21, δ_C 125.2 and 129.0) and a trisubstituted double bond (δ_H 5.63, δ_C 129.8 and 140.7) in the A/B rings. HMBC correlations from δ_H 2.70 (H-2β) and 2.51 (H-2α) to δ_C 212.9 (C-1) and 129.8, from δ_H 4.42 (H-3) to δ_C 129.8 and 140.7, from δ_H 5.63 to δ_C 48.2 (C-2), 66.3 (C-3), 49.9 (C-10), and 129.0, from δ_H 5.95 to δ_C 34.1 (C-9), and from δ_H 2.63 (H-8) to δ_C 129.0 and 125.2 indicated that the conjugated diene system was located at C-4 (δ_C 129.8) and C-5 (δ_C 140.7), C-6 (δ_C 129.0) and C-7 (δ_C 125.2). HMBC correlations from δ_H 5.33 (HO-3) to δ_C 48.2 (C-2), 66.3 (C-3), and 129.8 (C-4) and from δ_H 4.42 (H-3) to δ_C 129.8 (C-4), 140.7 (C-5), and 212.9 (C-1) confirmed that an OH group was at C-3. The NOESY spectrum of **2** (in DMSO-d_6) exhibited correlations between δ_H 0.92 (H-19) and δ_H 2.70 (H-2β), between δ_H 4.42 (H-3) and both δ_H 2.70 (H-2β) and 2.51 (H-2α), and between δ_H 5.33 (HO-3) and δ_H 2.51 (H-2α), supporting the α-orientation of the 3-OH group. Therefore, compound **2** was identified as 3α-hydroxy-2,3-dihydro-4,7-didehydrophysalin B and named physalin Z. The structure of **2** was similar to that of isophysalin G, except that the configuration at C-3 for isophysalin G was not established.[11] Comparison of the NMR data recorded in $CDCl_3$ demonstrated that **2** and isophysalin G should not be the same compound.

Compound **3** was obtained as a white powder ($C_{28}H_{30}O_{10}$). The NMR data (in DMSO-d_6) of **3** were very close to those of **2**, with differences in chemical shifts of the protons and carbons around C-3 indicating that **3** was the 3-epimer of **2**. This conclusion was supported by the HMBC data. The NOESY spectrum of **3** (in

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Table 1. ^{13}C NMR Data (δ) of Compounds **1**–**7**

position	**1**[a]	**1**[c]	**2**[b]	**2**[c]	**3**[b]	**3**[c]	**4**[b]	**5**[b]	**5**[c]	**6**[b]	**7**[b]
1	211.3	212.9	212.9	216.1	211.3	213.1	211.5	210.2	212.1	210.6	209.7
2	49.1	48.0	48.2	44.5	46.6	44.8	46.9	45.8	45.0	42.5	42.1
3	68.8	69.2	66.3	69.7	66.1	69.6	66.6	66.5	69.6	75.5	74.3
4	41.8	40.7	129.8	125.4	126.5	126.3	127.7	125.1	125.7	123.1	120.3
5	136.2	132.9	140.7	143.2	142.5	141.5	139.6	141.0	139.6	141.5	143.9
6	124.2	125.9	129.0	127.8	129.1	127.9	126.8	126.7	127.3	127.9	128.4
7	25.9	24.7	125.2	127.3	125.7	126.6	127.7	127.7	127.3	129.2	129.4
8	40.3	39.1	45.0	44.2	45.0	44.3	47.9	47.4	51.2	48.0	48.0
9	34.4	33.4	34.1	32.3	33.3	32.1	36.7	36.0	32.7	36.1	35.5
10	56.0	55.2	49.9	51.6	50.2	51.3	50.7	50.9	50.4	50.6	50.9
11	24.6	24.9	21.7	25.3	22.3	25.3	21.5	21.6	28.0	22.2	22.1
12	26.6	25.9	24.4	25.8	24.7	25.8	28.7	28.6	31.6	27.2	27.2
13	79.9	79.6	78.8	80.0	78.8	80.0	78.4	78.4	80.7	78.6	78.6
14	107.8	107.5	105.8	106.3	105.8	106.4	81.4	81.5	82.3	80.3	80.5
15	209.5	208.1	208.6	207.8	208.7	207.8	171.1	171.2	169.3	175.6	175.6
16	55.7	56.3	53.7	55.6	53.6	55.6	47.4	47.6	47.7	56.4	56.2
17	81.7	80.2	80.9	81.0	81.0	81.0	82.8	82.9	83.8	84.1	84.0
18	173.6	172.3	171.6	171.9	171.6	172.0	173.0	172.9	173.0	173.6	173.6
19	17.5	18.7	16.2	19.3	17.8	20.9	17.7	19.2	21.9	19.0	19.1
20	81.4	80.2	80.4	80.7	80.4	80.6	82.5	82.3	82.1	83.3	83.3
21	22.5	21.6	21.7	21.5	21.7	21.5	21.5	21.5	22.1	21.9	22.0
22	77.6	77.0	76.5	77.0	76.5	77.0	76.1	76.2	75.8	75.5	75.5
23	32.9	32.9	31.2	32.9	31.3	32.9	29.8	29.8	32.7	28.2	28.1
24	31.5	31.1	30.7	31.2	30.7	31.2	28.4	28.0	31.6	34.9	34.9
25	51.0	50.9	49.3	50.9	49.3	50.8	40.4	40.1	41.8	39.3	39.1
26	167.5	166.7	167.3	166.5	167.3	166.5	170.7	170.7	169.1	172.9	172.9
27	61.6	60.7	61.4	61.2	61.4	61.2	60.7	60.7	61.1	16.8	16.8
28	25.7	26.5	24.4	26.5	24.5	26.5	29.6	29.6	31.7	27.4	27.5
29										54.9	55.5

[a] C_5D_5N was used as solvent. [b] DMSO-d_6 was used as solvent. [c] $CDCl_3$ was used as solvent. The data of **1**[a], **3**[b], **4**[b], **6**[b], and **7**[b] were obtained at 75 MHz, while the data of **1**[c], **2**[b], **2**[c], **3**[c], **5**[b], and **5**[c] were obtained at 150 MHz.

Table 2. ^1H NMR Data (δ) of Compounds **1**–**3**

position	**1**[a]	**1**[c]	**2**[b]	**2**[c]	**3**[b]	**3**[c]
2	2.89 m	(α) 2.62 m	(α) 2.51 dd (12.2, 7.5)	(α) 2.50 m	2.62 m	(α) 2.87 m
	2.89 m	(β) 2.83 m	(β) 2.70 dd (12.2, 5.6)	(β) 3.43 dd (13.8, 7.2)	2.62 m	(β) 3.10 dd (12.2, 7.5)
3	4.06 m	3.77 m	4.42 m	4.93 m,	4.42 m,	4.67 m
4	2.66 m; 2.66 m	2.60 m; 2.53 m	5.63 br s	5.63 d (3.3)	5.72 d (4.9)	5.59 d (3.2)
6	5.53 br s	5.61 d (4.9)	6.21 dd (10.4, 2.8)	6.14 dd (10.3, 2.6)	6.21 dd (10.4, 2.7)	6.14 dd (10.4, 2.4)
7	2.51 m; 2.43 m	2.29 m; 2.14 m	5.95 br d (10.4)	6.21 br d (10.3)	5.97 dd (10.4, 1.4)	6.18 br d (10.4)
8	2.37 m	2.14 m	2.63 m	2.75 m	2.63 m	2.76 m
9	3.58 m	2.80 m	3.10 m	2.95 m	2.99 m	2.87 m
11	2.05 m; 1.47 m	1.60 m; 1.21 m	1.98 m; 0.87 m	1.62m; 1.25 m	1.82 m; 0.87 m	1.62 m; 1.25 m
12	2.62 m	2.24 m; 1.54 m	1.91 m; 1.35 m	2.32 m; 1.60 m	1.92 m; 1.40 m	2.32 m; 1.60 m
	1.89 dd (16.4, 10.5)					
16	3.11 s	2.20 s	2.85 s	2.20 s	2.86 s	2.19 s
19	1.19 s	1.28 s	0.92 s	1.31 s	1.07 s	1.40s
21	2.37 s	1.95 s	1.76 s	1.97 s	1.75 s	1.95 s
22	4.75 m	4.53 m	4.57 m	4.55 m	4.57 m	4.54 m
23	2.12 m	2.05 dd (14.7, 3.4)	2.11 dd (14.6, 3.6)	2.02 m	2.11 dd (14.5, 3.5)	2.07 m
	2.12 m	1.99 br d (14.7)	1.93 br d (14.6)	2.02 m	1.92 br d (14.5)	2.02 m
25	2.96 m	2.43 br d (4.1)	2.94 m	2.49 d (3.2)	2.94 m	2.49 d (4.4)
27	4.79 dd (13.4, 4.5)	4.49 dd (13.4, 4.6)	4.33 dd (13.5, 4.5)	4.62 dd (13.5, 4.6)	4.34 dd (13.5, 4.5)	4.61 dd (13.5, 4.6)
	3.99 d (13.4)	3.74 d (13.4)	3.65 d (13.5)	3.83 d (13.5)	3.66 d (13.5)	3.83 d (13.5)
28	1.31 s	1.24 s	1.15 s	1.28	1.16 s	1.28
HO-3			5.33 d (5.8)		5.21 d (5.0)	
HO-13			6.55 s		6.43 s	

[a] C_5D_5N was used as solvent. [b] DMSO-d_6 was used as solvent. [c] $CDCl_3$ was used as solvent. All the data were obtained at 600 MHz.

$CDCl_3$) exhibited NOE correlations between δ_H 1.40 (H-19) and δ_H 3.10 (H-2β) and between δ_H 4.67 (H-3) and δ_H 2.87 (H-2α), indicating a β-orientation of the OH group at C-3. Accordingly, **3** was deduced to be 3β-hydroxy-2,3-dihydro-4,7-didehydrophysalin B. The NMR data demonstrated that **3** and isophysalin G should be the same compound, and assignments of some carbon and proton signals for isophysalin G were revised on the basis of the ^1H, ^{13}C, HSQC, HMBC, and NOESY data.

Compound **4** was obtained as a yellowish powder ($C_{28}H_{30}O_{10}$). Comparison of the NMR data recorded in DMSO-d_6 demonstrated that **4** and physalin W were the same compound.[13] However, the NOESY spectrum of **4** displayed NOE correlations between δ_H 1.07 (H-19) and both δ_H 2.80 (H-2β) and 4.48 (H-3) and between δ_H 4.48 (H-3) and both δ_H 2.80 (H-2β) and 2.42 (H-2α), requiring the orientation of HO-3 to be α. Hence, the structure of physalin W (**4**) was revised to 3α-hydroxy-2,3-dihydro-4,7-didehydroneophysalin B.

Compound **5** was obtained as a yellowish powder ($C_{28}H_{30}O_{10}$). The NMR data demonstrated that **5** and physalin X should be the same compound,[13] the 3-epimer of **4**. Severe overlapping of the signals of H-2α and H-2β in the ^1H NMR spectrum (in DMSO-d_6) of **5** did not allow the determination of the configuration at C-3; thus, the ^1H, ^{13}C, HSQC, HMBC, and NOESY spectra of **5** were remeasured in $CDCl_3$. The NOESY spectrum (in $CDCl_3$) of **5** exhibited NOE correlations between δ 1.44 (H-19) and δ 3.03 (H-2β) and between δ 4.62 (H-3) and δ 2.86 (H-2α), supporting the

Table 3. ^{1}H NMR Data (δ) of Compounds **4**−**7**

position	**4**[a]	**5**[a]	**5**[b]	**6**[a]	**7**[a]
2	(α) 2.42 dd (12.4, 7.3)	(α) 2.63 m	(α) 2.86 dd (11.5, 7.2)	(α) 2.45 dd (12.1, 7.2)	(α) 2.61 m
	(*β*) 2.80 dd (12.4, 5.8)	(*β*) 2.63 m	(*β*) 3.03 dd (11.5, 8.5)	(*β*) 2.85 dd (12.1, 5.3)	(*β*) 2.61 m
3	4.48 m	4.37 m	4.62 m	4.20 m	4.08 m
4	5.55 d (2.0)	5.60 d (4.4)	5.58 br s	5.63 br s	5.77 d (5.1)
6	6.16 d (10.3)	6.15 dd (10.2, 2.2)	6.25 d (10.3)	6.27 d (10.2)	6.31 d (10.1)
7	6.06 dd (10.3, 2.7)	6.05 dd (10.2, 3.0)	6.16 dd (10.3, 3.0)	6.04 dd (10.2, 3.7)	6.04 dd (10.1, 3.8)
8	3.02 m	3.03 m	2.60 m	2.90 m	2.88 m
9	2.08 m	1.98 m	2.05 m	1.98 m	1.85 m
11	1.90 m; 1.52 m	1.87 m; 1.52 m	1.55 m; 1.42 m	1.81 m; 1.38 m	1.80 m; 1.35 m
12	2.20 m; 2.05 m	2.20 m; 2.06 m	2.05 m; 1.90 m	2.12 m; 2.12 m	2.12 m; 2.12 m
16	2.96 s	2.97 s	2.81 s	2.97 s	2.97 s
19	1.07 s	1.18 s	1.44 s	1.07 s	1.07 s
21	1.65 s	1.65 s	1.75 s	1.65 s	1.63 s
22	4.60 d (2.8)	4.59 dd (4.1, 1.4)	4.58 d (3.0)	4.44 d (2.1)	4.43 d (2.1)
23	2.04 dd (14.3, 3.9)	2.04 dd (15.0, 4.6)	2.03 m	2.01 dd (15.1, 4.0)	2.01 dd (15.1, 4.0)
	1.84 d (14.3)	1.82 d (15.0)	1.90 m	1.58 d (15.1)	1.58 d (15.1)
25	2.99 m	3.00 dd (11.9, 3.8)	2.57 m	3.44 d (7.5)	3.41 d (7.5)
27	3.94 dd (12.2, 4.0)	3.91 dd (12.2, 4.0)	4.48 dd (12.4, 3.3)	1.16 d (7.5)	1.16 d (7.5)
	4.31 t (12.2)	4.30 t (12.2)	4.32 t (12.4)		
28	1.36 s	1.36 s	1.46 s	1.25 s	1.24 s
	6.62 s (HO-13)	6.60 s (HO-13)		6.51 s (HO-13)	6.55 s (HO-13)
	5.26 d (5.3) (HO-3)	5.22 d (5.2) (HO-3)		6.23 s (HO-14)	6.22 s (HO-14)
				3.22 s (CH_3O-3)	3.20s (CH_3O-3)

[a] DMSO-d_6 was used as solvent. [b] $CDCl_3$ was used as solvent. All the data were obtained at 600 MHz.

α-orientation of H-3 and the *β*-orientation of the 3-OH group. Thus, the structure of physalin X (**5**) was revised to 3*β*-hydroxy-2,3-dihydro-4,7-didehydroneophysalin B.

Compound **6** was obtained as a yellowish powder ($C_{29}H_{34}O_{10}$). The ^{1}H NMR spectrum showed three tertiary methyl signals at δ_H 1.07 (H-19), 1.65 (H-21), and 1.25 (H-28). The ^{13}C NMR spectrum showed one ketone carbonyl signal at δ_C 210.6 (C-1), three lactone carbonyl signals at δ_C 175.6 (C-15), 173.6 (C-18), and 172.9 (C-26), four oxygenated quaternary carbon signals at δ_C 78.6 (C-13), 80.3 (C-14), 83.3 (C-20), and 84.1 (C-17), and one oxygenated methine carbon signal at δ_C 75.5 (C-22). These NMR data of **6** were similar to those of **4**, suggesting that **6** was also a neophysalin.[13] HMBC correlations placed the conjugated diene system at C-4 (δ_C 123.1) and C-5 (δ_C 141.5), C-6 (δ_C 127.9) and C-7 (δ_C 129.2). HMBC correlations from δ_H 3.22 to δ_C 75.5 (C-3) and from δ_H 4.20 (H-3) to δ_C 54.9, 123.1 (C-4), 141.5 (C-5), and 210.6 (C-1) placed the methoxy group at C-3. The ^{1}H NMR spectrum showed a secondary methyl group at δ_H 1.16 (H-27), and the HMBC correlations from δ_H 1.16 to δ_C 172.9 (C-26), 39.3 (C-25), and 34.9 (C-24) and from the proton of an OH group [δ_H 6.23(1H, s, HO-14)] to δ_C 175.6 (C-15) indicated that there was no C (14)−O−C (27) ether bridge. NOESY correlations between δ_H 1.07 (H-19) and both δ_H 2.85 (H-2*β*) and 4.20 (H-3), between δ_H 4.20 (H-3) and both δ_H 2.45 (H-2α) and 2.85 (H-2*β*), and between δ_H 3.22 (CH_3O-3) and δ_H 2.45 (H-2*β*) indicated that the C-3 methoxy group was α-oriented. Thus, **6** was assigned as 3α-methoxy-2,3,25,27-tetrahydro-4,7-didehydro-7-deoxyneophysalin A, and it was named physalin I.

Compound **7**, a yellowish powder, had the same molecular formula ($C_{29}H_{34}O_{10}$) as **6**. Comparison of the ^{1}H and ^{13}C NMR data of **7** with those of **6** suggested that they were 3-epimers. This conclusion was supported by the HMBC correlations from δ_H 3.20 (CH_3O-3) to δ_C 74.3 (C-3) and from δ_H 4.08 (H-3) to δ_C 55.5 (CH_3O-3), 120.3 (C-4), 143.9 (C-5), and 209.7 (C-1). NOESY correlations between δ_H 1.07 (H-19) and δ_H 2.61 (H-2*β*) and between δ_H 4.08 (H-3) and δ_H 2.61(H-2α) supported a *β*-orientation of the C-3 methoxy group. Therefore, the structure of **7** was assigned as 3*β*-methoxy-2,3,25,27-tetrahydro-4,7-didehydro-7-deoxyneophysalin A, and it was named physalin II.

In addition to four new physalins (**1**, **2**, **6**, **7**), 11 known steroidal compounds, isophysalin G (**3**),[11] physalin W (**4**),[13] physalin X (**5**),[13] physalin A (**8**),[14] physalin B (**9**),[14] physalin F (**10**),[15] physalin L (**11**),[16] physalin O (**12**),[14] physalin D (**13**),[17] physalin E (**14**),[18] and 4,7-didehydroneophysalin B (**15**),[11] as well as eight flavonoids, luteolin (**16**),[19] ombuine (**17**),[20] luteolin-7-*O*-*β*-*D*-glucopyranoside (**18**),[21] luteolin-4′-*O*-*β*-*D*-glucopyranoside (**19**),[22] quercetin-3-*O*-*β*-*D*-glucopyranoside (**20**),[23] quercetin-3,7-di-*O*-*β*-D-glucopyranoside (**21**),[24] luteolin-7,3′-*di*-*O*-*β*-D-glucopyranoside (**22**),[25] and luteolin-7,4′-di-*O*-*β*-D-glucopyranoside (**23**),[26] were also isolated and identified by comparison of their spectroscopic data with those reported in the literature.

Table 4. Inhibitory Effect of Compounds **1**–**23** on NO Production Induced by LPS in Macrophages[a]

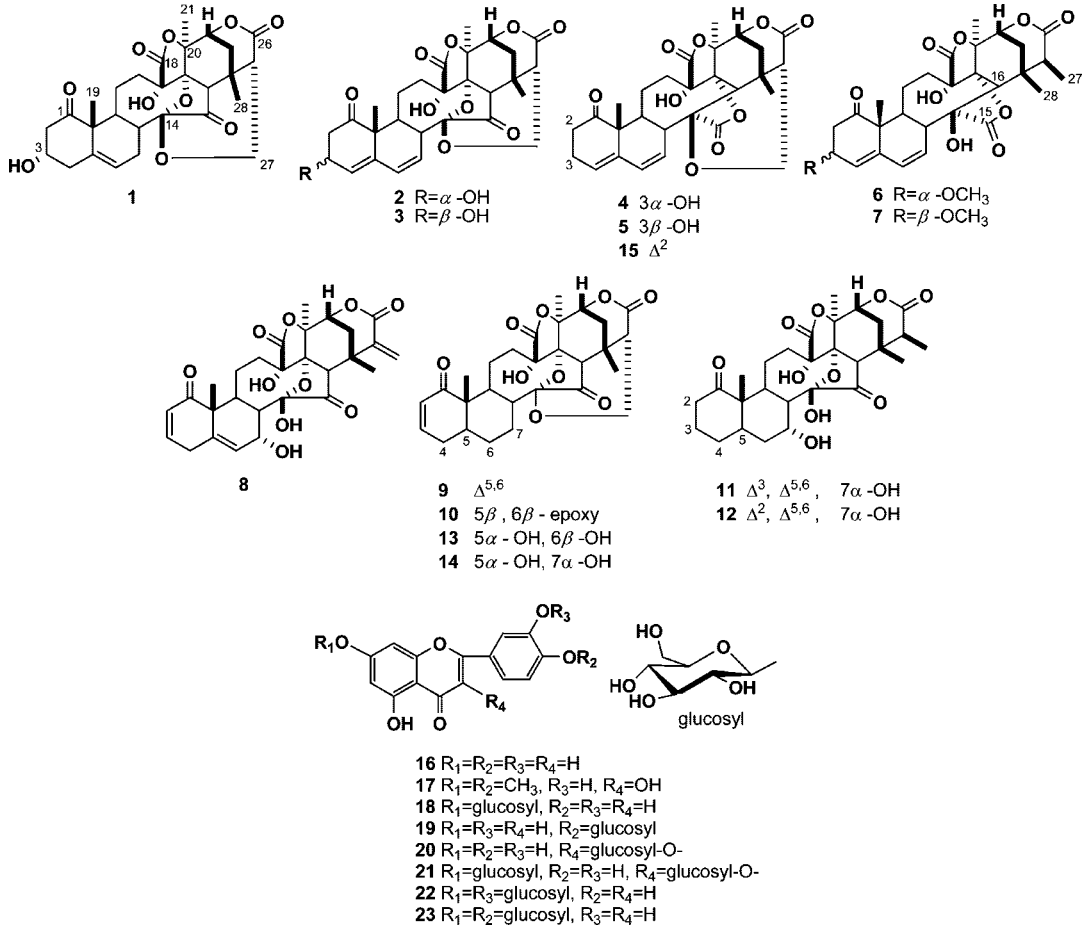
compound	$IC_{50} \pm$ SD (*μ*M)	compound	$IC_{50} \pm$ SD (*μ*M)
1	79.23 ± 7.81	**9**	0.84 ± 0.64
2	75.72 ± 4.68	**10**	0.33 ± 0.17
3	64.01 ± 7.64	**12**	17.11 ± 4.58
6	83.96 ± 5.51	**16**	7.39 ± 2.18
8	2.57 ± 1.18	**17**	2.23 ± 0.19
		hydrocortisone	64.34 ± 7.49

[a] NO concentration of control group: 3.96 ± 0.29 *μ*M. NO concentration of LPS-treated group: 34.78 ± 2.54 *μ*M.

Compounds **1**−**23** were examined for their inhibitory effects on NO production induced by LPS in macrophages (see Table 4). Cell viability in the present experiment was determined by the MTT method to find whether inhibition of NO production was due to cytotoxicity of test compounds (data not shown). As shown in Table 4, hydrocortisone (IC_{50} 64.3 ± 7.5 *μ*M) was used as a positive control. Compounds **9**, **8**, **10**, and **12** showed strong inhibition of NO production induced by LPS. Physalin F (**10**) possessed the strongest effect, with an IC_{50} of 0.33 ± 0.17 *μ*M. Compounds **1**−**3** exhibited moderate activities, which were close to that of hydrocortisone.

From the structural features of the physalin skeleton, it was found that the conjugated 2-en-1-one moiety at ring A and the epoxy group of C-5 and C-6 or the double bond between C-5 and C-6 at the B ring were important for activity (e.g., **1**, **8**, **9**, **10**, **12**). Although the conjugated 2-en-1-one moiety in the A ring still existed, the activity decreased dramatically when the B ring was saturated (e.g., **13**, **14**). Lack of a double bond between C-2 and C-3 decreased activity (e.g., **1**, **2**, **3**, **11**). Introduction of an OH or OCH_3 group at C-3 or C-7 led to a marked decrease of the activity (e.g., **1**, **2**, **3**, **8**, **11**, **12**, **14**). Conversion of the physalin skeleton to a neophysalin skeleton dramatically impaired the NO inhibitory effect (e.g., **4**, **5**, **6**, **7**, **15**).

Chart 1



Flavonoids **16**–**23** were also examined for their inhibitory effects on NO production induced by LPS in macrophages. Compounds **16** and **17** showed inhibition on LPS-induced NO production in macrophages with IC_{50} values of 7.4 and 2.2 μM, respectively. The flavonoid glucosides were inactive.

Experimental Section

General Experimental Procedures. Melting points (uncorrected) were measured on a Yanaco Mp-S3 micro melting point apparatus. Optical rotations were measured with a Perkin-Elmer 241 polarimeter. UV spectra were recorded with a Shimadzu UV 2201 spectrophotometer, and NMR experiments were performed on Bruker ARX-300 and 600 spectrometers, using TMS as an internal standard. ESIMS data were measured on an Agilent 1100-LC/MSDTrapSL mass spectrometer, and HRESIMS data were measured on an Agilent 6210 TOF mass spectrometer. Silica gel GF_{254} prepared for TLC and silica gel (200–300 mesh) for column chromatography (CC) were obtained from Qingdao Marine Chemical Company (Qingdao, PR China). Polyamide film (10 cm × 10 cm) for TLC and polyamide (80–140 mesh) for CC were supplied by Taizhou Luqiao Sijia Biochemical Plastics Company (Zhejiang, PR China). Sephadex LH-20 was a product of Pharmacia Co. Rp-18 (40–75 mm) silica gel was purchased from Merk Chemical Ltd. Preparative HPLC (PHPLC) was carried out on a Waters 600 apparatus with a preparative reversed-phase C18 column (Inertsil Prep-ODS 20 mm × 250 mm, 10 μm, GL Sciences Inc.).

Plant Material. The calyces of *Phsalis alkekengi* var. *franchetii* were collected in September 2004, near Yilan, Heilongjiang Province, PR China, and were authenticated by Prof. Qishi Sun, Department of Pharmaceutical Botany, School of Traditional Chinese Materia Medica, Shenyang Pharmaceutical University. A voucher specimen (collection number: 20041019) has been deposited in the herbarium of the laboratory of natural products chemistry, Shenyang Pharmaceutical University.

Extraction and Isolation. Dried calyces (5 kg) of *P. alkekengi* var. *franchetii* were extracted with 80% EtOH under reflux, and about 800 g of extract was obtained. The extract was suspended in water and then partitioned with cyclohexane, EtOAc, and *n*-BuOH successively. The EtOAc extract was evaporated under vacuum (120 g). A portion of the EtOAc extract (80 g) was chromatographed on a silica gel column using a gradient of $CHCl_3-CH_3OH$ (100:1 to 0:100), which yielded nine fractions (1–9). Fraction 1 was chromatographed over silica gel using a gradient of cyclohexane−EtOAC (100:1 to 0:100) to obtain nine subfractions (11–19). Fractions 16, 17, and 18 were recrystallized from acetone to yield **9** (800 mg), **10** (650 mg), and **8** (1.5 g), respectively. Fraction 2 was separated by PHPLC using $MeCN-H_2O$ (50:50) to yield **11** (11 mg) and **12** (33 mg), respectively. The mother liquid of fraction 2 was chromatographed on silica gel using cyclohexane−EtOAC (100:1 to 0:100) to afford nine subfractions (21–29). Fraction 28 was submitted to Sephadex LH-20 ($CHCl_3-CH_3OH$, 1:1) to yield fractions 281–283. Fraction 282 was subjected to PHPLC ($MeCN-H_2O$) followed by preparative TLC (PTLC) using cyclohexane−EtOAC (1:2) to yield **2** (20 mg). CC of fraction 3 resulted in nine subfractions (31–39). Fraction 35 was applied to Sephadex LH-20 ($CHCl_3-CH_3OH$, 1:1) to yield three subfractions (351–353), and fractions 351 and 352 were separated by PHPLC ($MeCN-H_2O$, 45:55) to afford **6** (5.5 mg), **7** (5.0 mg), and **15** (47 mg). Fraction 352, same procedure, yielded **5** (5.1 mg). Fraction 353 was crystallized from MeOH to yield **17** (16 mg). Fraction 36 was separated on Sephadex LH-20 ($CHCl_3-CH_3OH$, 1:1) to afford subfractions 361–363. Fraction 362, same procedure as 351, yielded **4** (5.1 mg) and fraction 3622. Purification of fraction 3622 by PTLC (cyclohexane−EtOAC, 1:2) yielded **3** (18 mg). The crude crystals of fraction 4 were recrystallized (acetone) to obtain **13** (200 mg). The mother liquid of fraction 4 was applied to Sephadex LH-20 ($CHCl_3-CH_3OH$, 1:1) to afford three subfractions (41–43). Fraction 42 was separated by PHPLC ($MeCN-H_2O$, 45:55) and crystallized from acetone to give **14** (30 mg). Fraction 43 was crystallized from MeOH to yield **16** (20 mg). Fraction 6 was chromatographed on polyamide eluted with $H_2O-MeOH$ (100:0 to 0:100). The $H_2O-MeOH$ (50:50) eluent was concentrated and submitted to Sephadex LH-20 ($MeOH-H_2O$, 1:1) to yield fractions 641–644. Fraction 643 was

subjected to RPCC eluted with H_2O−MeOH (100:0 to 0:100) to yield subfractions 6431–6436. Fraction 6434 yielded **1** (37 mg).

The *n*-BuOH extract (100 g) was chromatographed on polyamide [H_2O−MeOH (100:0 to 0:100)] to yield fractions B1–B6. Fraction B3 was separated on Sephadex LH-20 (MeOH−H_2O, 1:1) to yield subfractions B31–B33. Fraction B32 was subjected to PHPLC, using MeOH−H_2O−HAc (30:70:0.1), to give **21** (40 mg), **22** (14 mg), and **23** (16 mg). Fraction B5 was crystallized from MeOH to yield **18** (1.6 g), and the mother liquid was separated on Sephadex LH-20 CC (MeOH−H_2O, 1:1) to yield subfractions B51–B52. Fraction B52 was separated by PHPLC, using MeOH−H_2O−HAc (45:55:0.1), to give **19** (10 mg) and **20** (27 mg).

Physalin Y (1): white powder, mp 281–282 °C; $[\alpha]^{20}_D$ −102.9 (*c* 0.11, MeCN); UV (MeCN) λ_{max} (log ϵ) 193 (4.1) nm; ^{1}H NMR (C_5D_5N, 600 MHz) Table 2 and ^{13}C NMR (C_5D_5N, 75 MHz) Table 1; ^{1}H NMR ($CDCl_3$, 600 MHz) Table 2 and ^{13}C NMR ($CDCl_3$, 150 MHz) Table 1; ESIMS *m*/*z* 527 $[M - H]^-$, 563 $[M + Cl]^-$; HRESIMS *m*/*z* 551.1891 $[M + Na]^+$ (calcd for $C_{28}H_{32}O_{10}Na$, 551.1893).

Physalin Z (2): white powder, mp 234–235 °C; $[\alpha]^{20}_D$ −67.5 (*c* 0.12, MeCN); $[\alpha]^{20}_D$ −25 (*c* 0.19, $CHCl_3$); UV (MeCN) λ_{max} (log ϵ) 230 (4.1) nm; ^{1}H NMR (DMSO-d_6, 600 MHz) Table 2 and ^{13}C NMR (DMSO-d_6, 150 MHz) Table 1; ^{1}H NMR ($CDCl_3$, 600 MHz) Table 2 and ^{13}C NMR ($CDCl_3$, 150 MHz) Table 1; ESIMS *m*/*z* 561 $[M + Cl]^-$, 525 $[M - H]^-$, 571 $[M + HCOO^-]^-$, 549 $[M + Na]^+$; HRESIMS *m*/*z* 549.1729 $[M + Na]^+$ (calcd for $C_{28}H_{30}O_{10}Na$, 549.1737).

Isophysalin G (3): white powder, mp 237–238 °C; $[\alpha]^{20}_D$ −94.6 (*c* 0.11, MeCN); $[\alpha]^{20}_D$ −31 (*c* 0.10, $CHCl_3$); UV (MeCN) λ_{max} (log ϵ) 229 (4.2) nm; ^{1}H NMR (DMSO-d_6, 600 MHz) Table 2 and ^{13}C NMR (DMSO-d_6, 75 MHz) Table 1; ^{1}H NMR ($CDCl_3$, 600 MHz) Table 2 and ^{13}C NMR ($CDCl_3$, 150 MHz) Table 1; ESIMS *m*/*z* 561 $[M + Cl]^-$, 525 $[M - H]^-$, 549 $[M + Na]^+$; HRESIMS *m*/*z* 549.1730 $[M + Na]^+$ (calcd for $C_{28}H_{30}O_{10}Na$, 549.1737).

Physalin W (4): yellowish powder; $[\alpha]^{20}_D$ −20.5 (*c* 0.095, MeOH); UV (MeCN) λ_{max} (log ϵ) 232 (4.0) nm; ^{1}H NMR (DMSO-d_6, 600 MHz) Table 3 and ^{13}C NMR (DMSO-d_6, 75 MHz) Table 1; HRESIMS *m*/*z* 549.1740 $[M + Na]^+$ (calcd for $C_{28}H_{30}O_{10}Na$, 549.1737).

Physalin X (5): yellowish powder; $[\alpha]^{20}_D$ −63.0 (*c* 0.10, MeOH); UV (MeCN) λ_{max} (log ϵ) 231 (4.1) nm; ^{1}H NMR (DMSO-d_6, 600 MHz) Table 3 and ^{13}C NMR (DMSO-d_6, 150 MHz) Table 1; HRESIMS *m*/*z* 549.1737 $[M + Na]^+$ (calcd for $C_{28}H_{30}O_{10}Na$, 549.1737).

Physalin I (6): yellowish powder, mp 254–255 °C; $[\alpha]^{20}_D$ −53.7 (*c* 0.095, MeOH); UV (MeCN) λ_{max} (log ϵ) 243 (4.0) nm; ^{1}H NMR (DMSO-d_6, 600 MHz) Table 3 and ^{13}C NMR (DMSO-d_6, 75 MHz) Table 1; HRESIMS *m*/*z* 543.2224 $[M + H]^+$ (calcd for $C_{29}H_{35}O_{10}$, 543.2230).

Physalin II (7): yellowish powder, mp 237–238 °C; $[\alpha]^{20}_D$ −144.7 (*c* 0.075, MeOH); UV (MeCN) λ_{max} (log ϵ) 242 (4.0) nm; ^{1}H NMR (DMSO-d_6, 600 MHz) Table 3 and ^{13}C NMR (DMSO-d_6, 75 MHz) Table 1; HRESIMS *m*/*z* 565.2047 $[M + Na]^+$ (calcd for $C_{29}H_{34}O_{10}Na$, 565.2050).

Biossay for NO Production. Mouse monocyte-macrophage RAW 264.7 cells (ATCC TIB-71) were purchased from the Chinese Academy of Science. RPMI 1640 medium, penicillin, streptomycin, and fetal bovine serum were purchased from Invitrogen (New York). Lipopolysaccharide (LPS), dimethylsufoxide (DMSO), 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyltetrazolium bromide (MTT), and hydrocortisone were obtained from Sigma Co. RAW 264.7 cells were suspended in RPMI 1640 medium supplemented with penicillin (100 U/mL), streptomycin (100 μg/mL), and 10% heat-inactivated fetal bovine serum. The cells were harvested with trypsin and diluted to a suspension in fresh medium. The cells were seeded in 96-well plates with 1×10^5 cells/well and allowed to adhere for 2 h at 37 °C in 5% CO_2 in air. Then, the cells were treated with 1 μg/mL of LPS for 24 h with or without various concentrations of test compounds. DMSO was used as a solvent for the test compounds, which were applied at a final concentration of 0.2% (v/v) in cell culture supernatants. NO production was determined by measuring the accumulation of nitrite in the culture supernatant using Griess reagent.[27] Briefly, 100 μL of the supernatant from incubates was mixed with an equal volume of Griess reagent (0.1% *N*-[1-naphthyl]ethylenediamine and 1% sulfanilamide in 5% H_3PO_4). Cytotoxicity was determined by the MTT colorimetric assay, after 24 h incubation with test compounds. The concentration of NO_2^- was calculated by a working line from 0, 1, 2, 5, 10, 20, 50, and 100 μM sodium nitrite solutions, and the inhibitory rate on NO production induced by LPS was calculated by the NO_2^- levels as follows:

$$\text{Inhibitory rate (\%)} = 100 \times \frac{[NO_2^-]_{LPS} - [NO_2^-]_{LPS+sample}}{[NO_2^-]_{LPS} - [NO_2^-]_{untreated}} \quad (1)$$

Experiments were performed in triplicate, and data are expressed as the mean ± SD of three independent experiments.

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