New Dibenzocyclooctadiene Lignans from *Schisandra sphenanthera* and Their Pro-inflammatory Cytokine Inhibitory Activities

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Investigation of the fruits of *Schisandra sphenanthera* led to the isolation of two new dibenzocyclooctadiene lignans, methylgomisin O (**1**) and chloromethyl schisantherin B (**2**), together with twelve known lignans (**3** – **14**). Their structures were elucidated by using extensive spectroscopic techniques including 1D and 2D NMR spectra. Compound **2** was identified as a cyclooctadiene moiety substituted with a chloromethyl group, which is rarely found in natural products, especially in terrestrial higher plants. Among these isolates, compounds **1** and **7** exhibited considerable inhibitory activity against tumor necrosis factor-α (TNF-α) and interleukin-6 (IL-6) production, and did not display any cellular toxicity against RAW264.7 cells.

Key words: *Schisandra sphenanthera*, Dibenzocyclooctadiene Lignans, Methylgomisin O, Chloromethyl Schisantherin B, Anti-inflammatory Activity

Introduction

Approximately 150 lignan derivatives possessing the dibenzocyclooctadiene skeleton have been isolated from plants of the schizandraceae family [1, 2], and a wide variety of biological activities exhibited by these lignans have been uncovered. Extracts from lignan-rich plants have been used in Chinese traditional medicine as tonics, sedatives, and astringent agents [3]. In addition to insecticidal [4 – 7] and antifeedant activity [8], dibenzocyclooctadiene lignans from Schizandraceae have been reported to inhibit cyclic-AMP phosphodiesterases. Several lignan derivatives inhibit the binding of the platelet activating factor to receptors on platelets [9 – 11]. A number of other derivatives suppress the proliferation of human peripheral blood lymphocytes and, thus, may be useful as immunosuppressive agents [12]. Several lignans exhibit significant biological activity, both *in vitro* and *in vivo* against carbon tetrachloride- and galactosamine-induced liver damage in different animal models. (–)-Wuweizisu C is considered a crucial component for the antihepatotoxic activity found in traditional Chinese medicine formulations of wuweizi lignan-containing plants [13 – 26]. Lignans also exhibit inhibitory activity against viral reverse transcriptase. For example, gomisin-G exhibited potent anti-HIV activity with an EC_{50} of 0.006 μg mL^{-1} and a therapeutic index (TI) of 300, while schizantherin D, kadsuranin, and (–)-wuweizisu C showed good activities with EC_{50} values of 0.5, 0.8, and 1.2 μg mL^{-1} and TI values of 110, 56, and 33.3, respectively. The results with these natural lignans have suggested that 9-benzoyl and 8-hydroxy substituents might enhance the biological activity [27 – 30].

However, little work on the isolation and characterization of anti-inflammatory constituents from this family has been done. As a part of our research program to find new compounds with anti-inflammatory activities from natural sources, the phytochemical investigation of *Schisandra sphenanthera* has been carried out. Repeated column chromatography of an EtOAc extract of *S. sphenanthera* led to the isolation of two new dibenzocyclooctadiene lignans, methylgomisin O (**1**) and chloromethyl schisantherin B (**2**), as well as twelve known ones, epigomisin O (**3**) [31], 6-*O*-benzoylgomisin (**4**) [32], gomisin J (**5**) [33], methylgomisin R (**6**) [34], schisantherins A – D (**7** – **10**) [35], (+)-deoxyschisandrin (**11**) [33], (+)-gomisin K_3 (**12**) [36], myristargenol A (**13**) [37], and sphenanlignan (**14**) [38].

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Table 1. ^{1}H and ^{13}C NMR spectral data of **1** and **2** (δ in ppm, J in Hz).

	— **1**[a,b] —		— **2**[c,d] —	
	δ_C	δ_H	δ_C	δ_H
1	151.3 (s)		153.0 (s)	
2	141.2 (s)		142.6 (s)	
3	151.9 (s)		152.8 (s)	
4	111.1 (d)	6.53 (s)	110.2 (d)	7.26 (s)
5	133.8 (s)		132.4 (s)	
6	90.1 (d)	3.87[f]	81.6 (d)	6.06 (s)
7	38.6 (d)	1.83 – 1.86 (m)	75.8 (s)	
8	36.5 (d)	1.67 – 1.71 (m)	43.2 (d)	2.34 – 2.37 (m)
9	37.5 (t)	2.25 – 2.30 (m)	36.8 (t)	2.34 – 2.37[f]
		1.94 – 2.03 (m)		2.22 – 2.25 (m)
10	134.2 (s)		135.4 (s)	
11	102.3 (d)	6.39(s)	102.7 (d)	6.55 (s)
12	148.5 (s)		149.7 (s)	
13	135.1 (s)		136.0 (s)	
14	142.0 (s)		141.7 (s)	
15	121.5 (s)		122.0 (s)	
16	123.7 (s)		123.0 (s)	
17	17.1 (q)	0.89 (d. 6.76)	18.8 (q)	1.20 (d. 9.3)
18	17.2 (q)	0.88 (d. 6.99)		
1-OMe	60.9 (q)	3.54 (s)	60.7 (q)	3.45 (s)
2-OMe	60.3 (q)[e]	3.87 (s)[e]	60.7 (q)	3.81 (s)
3-OMe	56.0 (q)	3.93 (s)	56.1 (q)	3.91 (s)
OCH_2O	100.6 (t)	5.95. 5.91 (each s)	101.5 (t)	5.95. 5.90 (each ABd. 0.8)
CH_2Cl			48.3 (t)	3.86. 3.53 (each ABd. 11.8)
6-OMe	55.7 (q)	3.03 (s)		
14-OMe	59.4 (q)[e]	3.91 (s)[e]	59.1 (q)	3.68 (s)
OH				4.03 (brs)
1′			166.2 (s)	
2′			128.5 (s)	
3′			139.5 (d)	5.98 (m)
4′			19.8 (q)	1.36 (brs)
5′			15.8 (q)	1.81 (dd. 7.2. 1.5)

[a] Measured in $CDCl_3$; [b] measured at 500 MHz; [c] measured in $(CD_3)_2CO$; [d] measured at 400 MHz; [e] assignments may be interchanged; [f] overlapped.

Results and Discussion

Compound **1** was isolated as a colorless amorphous powder, and its molecular formula of $C_{24}H_{30}O_7$ was established on the basis of HR-ESI-MS analysis ($[M+Na]^+$, m/z = 453.1881) and its ^{13}C NMR spectrum, indicating 10 degrees of unsaturation. The 1D NMR spectrum of **1** revealed that it possessed a biphenyl moiety due to two aromatic protons at δ_H = 6.53 and 6.39 (4-H and 11-H) and twelve aromatic carbon atoms (δ_C = 151.9, 151.3, 148.5, 142.0, 141.2, 135.1, 134.2, 133.8, 123.7, 121.5, 111.1, 102.3) (see Table 1), which indicated that **1** was a dibenzocyclooctadiene lignan.

The ^{1}H NMR spectrum of **1** displayed signals for four methoxy groups (δ_H = 3.93, 3.91, 3.87, and 3.54) and one methylenedioxy group (δ_H = 5.95 and 5.91) as substituents on the biphenyl rings. Moreover, in the cyclooctadiene ring, two secondary methyl groups [δ = 0.88 (d, J = 6.99 Hz, 18-Me), 0.89 (d, J = 6.76 Hz, 17-Me)] are placed at C-7 and C-8, respectively, which could be confirmed by the HMBC correlations from Me-18 to C-8, C-7 and C-6 and from Me-17 to C-7, C-8 and C-9. In addition, a benzylic methylene group [δ = 2.25 – 2.30 (m, 9-H), 1.94 – 2.03 (m, 9-H)], a benzylic oxymethine [δ = 3.87 (overlapped, 6-H)] and two methine groups at δ = 1.84 (m, 7-H) and δ = 1.67 (m, 8-H) were also found at the cyclooctadiene ring.

Comparison of the ^{1}H and ^{13}C NMR data between **1** and gomisin O [31] indicated that the substitution pattern on their biphenyl moieties was identical. The only difference was the presence of one more methoxy group (δ_H = 3.03) in **1**, instead of the hydroxy group at C-6 in gomisin O. The HMBC correlations (Fig. 1) of the methoxy group at δ = 3.03 with C-6 and of δ = 3.87 (overlapped, 6-H) with the aromatic carbon C-4

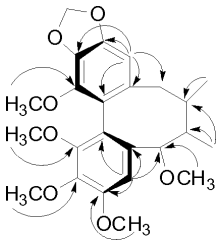


Fig. 1. Key HMBC correlations of compound **1**.

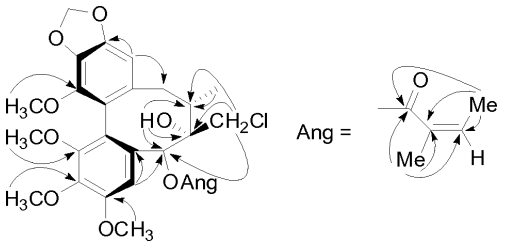


Fig. 2. Key HMBC correlations of compound **2**.

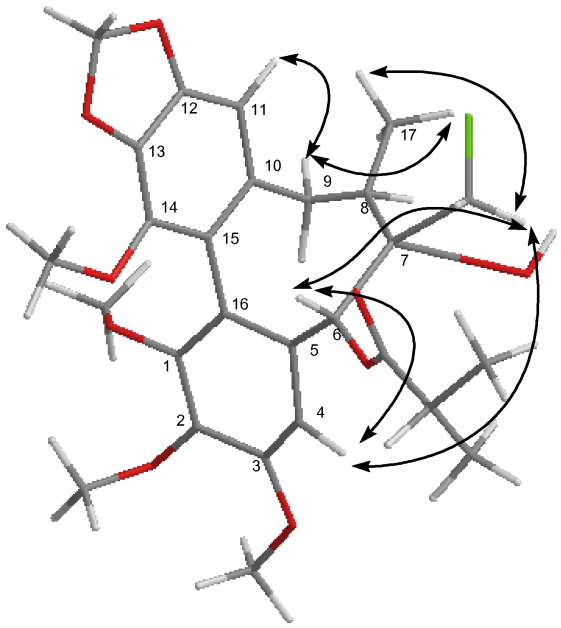


Fig. 3. Key ROESY correlations of compound **2**.

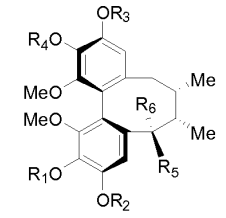
further supported this conclusion. Thus, **1** was a methyl derivative of gomisin O.

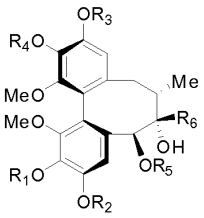
The ROESY spectrum of **1** showed cross-peaks between 17-Me and 4-H, 17-Me and 6-H, indicating that **1** possesses a twist-boat (TB) conformation of the cyclooctadiene ring. The similar chemical shifts of 17-Me and 18-Me further confirmed this conclusion [18, 27, 33]. A correlated peak between 4-H and 6-H indicated that the methoxy group at C-6 was located in β-position [39, 40]. The CD spectrum of **1** exhibited a positive Cotton effect around 210 – 231 nm and a negative Cotton effect around 250 – 255 nm, suggesting that **1** possesses an *S*-biphenyl configuration [31].

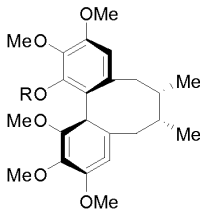
The EI-MS of **2** gave a molecular ion peak at *m/z* = 548, accompanied by an isotopic peak at *m/z* = 550 (their relative abundance ratio was 3 : 1), suggesting the presence of a chlorine atom [41], which was further supported by the fragment at *m/z* = 512 $[M–HCl]^+$. Its HR-ESI-MS [*m/z* = 571.1703, $[M+Na]^+$ (calcd. 571.1710 for $C_{28}H_{33}O_9ClNa$)] further revealed the molecular formula as $C_{28}H_{33}O_9Cl$. In addition, the elemental analytical results of **2** (anal. calcd. for $C_{28}H_{33}O_9Cl{\cdot}1/2H_2O$: C 60.27; H 6.14; found C 59.50; H 6.03) showed good agreement with the calculated values.

The ^{1}H NMR spectrum of **2** showed signals of two aromatic protons [δ = 7.26 (s, 4-H), 6.55 (s, 6-H)] of a biphenyl moiety. The signals at [δ = 3.45, 3.68, 3.81, 3.91 (each s, 4 × -OCH_3), and δ = 5.90, 5.95 (each ABd, *J* = 0.8 Hz, -OCH_2O-) indicated the presence of four methoxy and one methylenedioxy groups as substituents on the biphenyl rings. Moreover, one hydroxy at δ = 4.03 (s, -OH) and one secondary methyl at δ = 1.20 (d, *J* = 9.3 Hz, 17-Me) were also observed in the cyclooctadiene ring. The ^{13}C NMR spectral data of **2** indicated the presence of an angeloyl group (δ_C = 166.2, 139.5, 128.5, 19.8 and 15.8), which was further supported by the significant peaks at *m/z* = 83 $[MeCH=C(Me)CO]^+$ and 55 $[MeCH=CMe]^+$ in the EI-MS. The HMBC correlation (Fig. 2) observed between the proton at δ = 6.06 (s, 6-H) and the carbonyl group at δ = 166.2 (C-1′) suggested that the angeloyl group was attached to C-6.

Careful analysis of the ^{1}H and ^{13}C NMR spectral data of compound **2** suggested that this compound also was a dibenzocyclooctadiene lignan and similar to schisantherin B (**8**) [35] concerning the biphenyl group and the cyclooctadiene moiety including their substituents. The only difference between the two compounds was that a chloromethyl group in **2** replaced the methyl group in **8** at C-7. The presence of the chloromethyl group was indicated by the signals at δ = 3.53, 3.86 (each ABd, *J* = 11.8 Hz, CH_2Cl) in the ^{1}H NMR and at δ_C = 48.3 in the ^{13}C NMR spectrum (Table 1). In addition, HMBC correlations from two protons at δ_H = 3.86 and 3.53 to C-6, C-7 and C-8 further confirmed that the chloromethyl was located at C-7. Therefore, **2** was a chlorinated derivative of schisantherin B.







1* R_1=R_2=Me, R_3+R_4=CH_2, R_5=OMe, R_6=H

2* R_1=R_2=Me, R_3+R_4=CH_2, R_5=Ang, R_6=CH_2Cl

11 R=H

3 R_1=R_2=Me, R_3+R_4=CH_2, R_5=H, R_6=OH

7 R_1=R_2=Me, R_3+R_4=CH_2, R_5=Bz, R_6=Me

12 R=Me

4 R_1=R_2=Me, R_3+R_4=CH_2, R_5=OBz, R_6=H

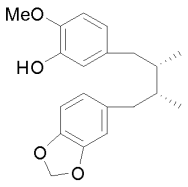
8 R_1=R_2=Me, R_3+R_4=CH_2, R_5=Ang, R_6=Me

5 R_1=R_4=Me, R_2=R_3=H, R_5=R_6=H

9 R_1=R_2=Me, R_3+R_4=CH_2, R_5=Tig, R_6=Me

6 R_1+R_2=CH_2, R_3+R_4=CH_2, R_5=OMe, R_6=H

10 R_1+R_2=CH_2, R_3+R_4=CH_2, R_5=Bz, R_6=Me



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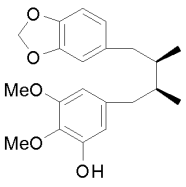

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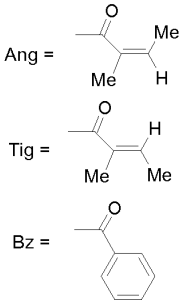

The stereochemistry of the cyclooctadiene moiety was elucidated by the ROESY spectrum (Fig. 3). The ROESY correlation observed between 4-H and 6-H suggested the α-orientation of 6-H. Therefore, the angeloyl group was located on the β-face of the molecule. The NOE interactions between 6-H and 7-OH, 7-OH and 17-Me designated the α-orientation of hydroxyl at C-7 and methyl at C-8. This was further evident from the significant NOE interaction between 9-Hα and 17-Me. Therefore, the chloromethyl group at C-7 was in a β-orientation. Further, the ROESY correlations between 4-H and 6-H, between Me-17 and 9-H, as well as between 9-H and 11-H indicated a twist-boat-chair (TBC) conformation of the cyclooctadiene ring [42]. Besides, **2** showed negative Cotton effects around 255 nm and positive effects around 220–232 nm in the CD spectrum, indicating that **2** possesses an *S*-biphenyl configuration. Therefore, the complete structure of **2** was determined to be that shown in Fig. 2, and named as chloromethyl schisantherin B.

Naturally occurring halogenated compounds have been isolated mainly from the marine environment and microorganisms [43]. Many chlorine- and bromine-containing metabolites from marine source have been investigated because of recent interest in their various biological activities as well as in their structural diversity [44]. However, only a few organochlorine compounds have been isolated from terrestrial higher plants [45]. Considering the distinct differences in environmental factors between the land and the sea, the limited occurrence of halogenated compounds in terrestrial higher plants is not surprising. This is the first example of chlorinated metabolites from the family Schisandraceae [46]. However, the isolation of **2** from the fruits of *Schisandra sphenanthera* posed a significant question about the origin of the chlorine atom.

We could not examine the biosynthetic origin due to the small quantity of **2**. We speculate that chloromethyl schisantherin B was derived from schisantherin B by induced enzymatic chlorination in the plant under stress conditions [43].

The known compounds were determined to be epigomisin O (**3**), 6-*O*-benzoylgomisin (**4**), gomisin J (**5**), methylgomisin R (**6**), schisantherins A–D (**7**–**10**), (+)-gomisin K_3 (**11**), (+)-deoxyschisandrin (**12**), myristargenol A (**13**) and sphenanlignan (**14**) by comparison of their spectral data with those of the literature.

The effects of compounds **1** and **7** on lipopolysaccharide (LPS)-induced TNF-α and interleukin-6 (IL-6) production in RAW264.7 macrophages were investigated. As is known, macrophage activation plays

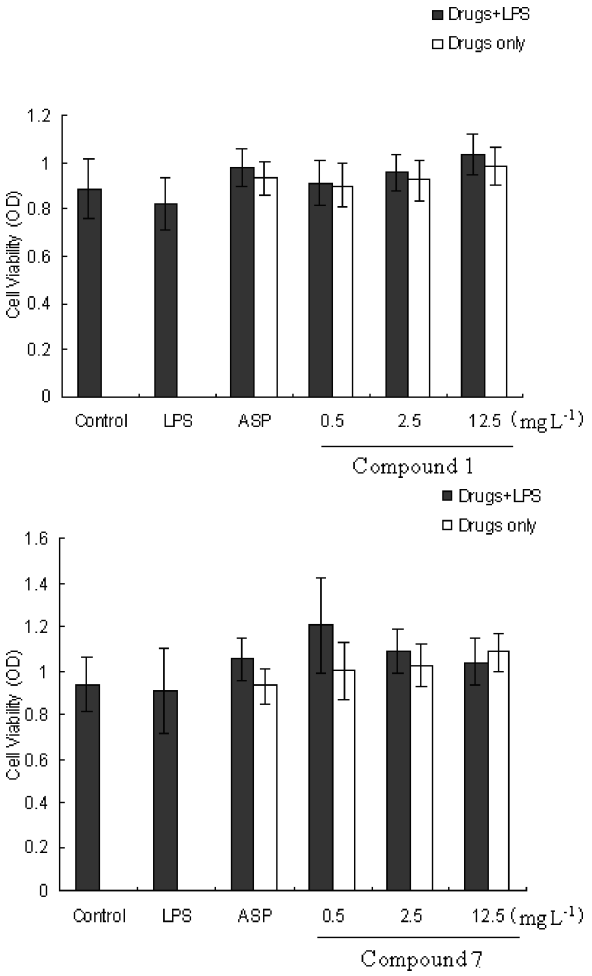

Fig. 4. The respective effects of **1** and **7** on the viability of RAW264.7 cells.

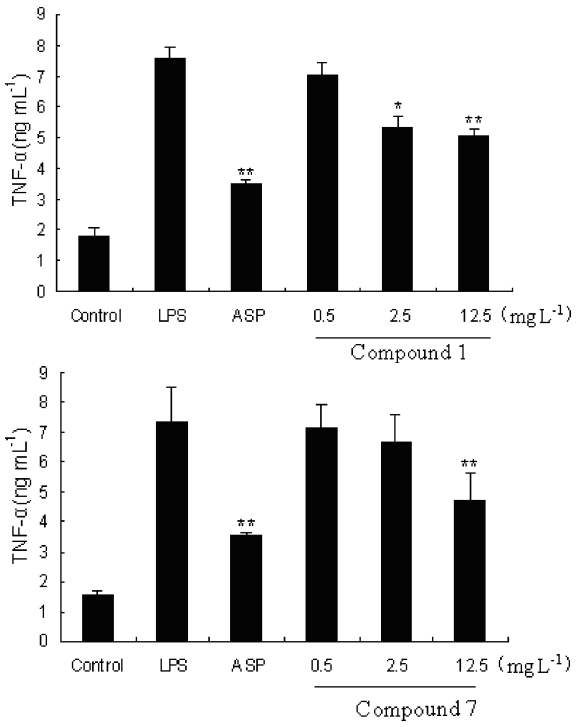

Fig. 5. The respective effects on the secretion of TNF-α *in vitro* for **1** and **7**.

an important role in the inflammatory process and produces potent proinflammatory cytokines such as TNF-α and IL-6 [47]. TNF-α is one of the most important cytokines, and is required for the synergistic induction of NO synthesis in LPS-stimulated macrophages. Moreover, TNF-α elicits a number of physiological effects, such as septic shock, inflammation, cachexia, and cytotoxicity [48]. IL-6 also is one of the most important inflammatory cytokines, and its production is induced by several factors, such as TNF-α, IL-1β as well as LPS. IL-6 is believed to be an endogenous mediator of LPS-induced fever in addition to its multiple effects on the immune system and hematopoiesis [49]. Recently, new approaches for the use of Chinese herbal plants to prevent and treat inflammatory responses by inhibiting inflammatory cytokines, such as TNF-α, IL-6, and IL-1β have become an important area of investigation [47].

In our present study, the anti-inflammatory potential and cellular toxicity of compounds **1** and **7** were tested, using the method previously described [50]. Compounds **1** and **7** did not display cellular toxicity against RAW264.7 cells over 20 h, as determined by the MTT assay (Fig. 4). TNF-α and IL-6 concentrations in the culture supernatants of RAW264.7 cells were measured by sandwich ELISA. Compared to the LPS control group, the concentration of TNF-α decreased significantly at 2.5 and 12.5 mg L^{-1} ($^{*}P < 0.05$, $^{**}P < 0.01$) with **1** or **7** pretreatment, respectively (Fig. 5). Moreover, IL-6 levels decreased from 0.5 to 12.5 mg L^{-1} ($^{*}P < 0.05$, $^{**}P < 0.01$) with on pretreatment with compound **1** or **7** (Fig. 6).

The present study demonstrated that compounds **1** and **7** inhibit the production of TNF-α and IL-6 in a dose-dependent manner in LPS-stimulated RAW264.7 cells. It also suggests that compounds **1** and **7** are bioactive lignans in *S. sphenanthera*, and thus their regulating effect on TNF-α and IL-6 formation might be associated with its putative anti-inflammatory effect. Future studies will focus on the basic mecha-

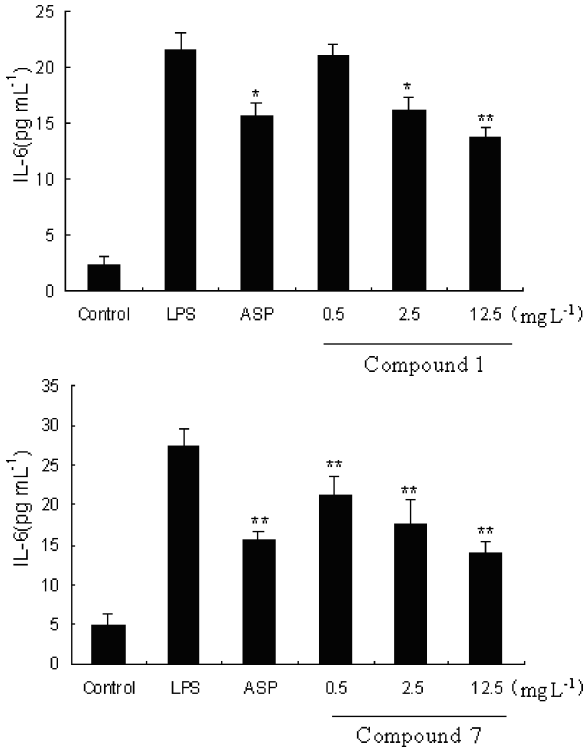

Fig. 6. The respective effects on the secretion of IL-10 *in vitro* for **1** and **7**.

nisms governing compounds' **1** and **7** inhibition of pro-inflammatory cytokine production.

Experimental Section

General experimental procedures

Optical rotations were measured with a Jasco DIP-370 digital polarimeter. CD spectra were measured with a JASCO J-810 spectropolarimeter. 1D and 2D NMR spectra were recorded using Bruker AM-400 and DRX-500 instruments with tetramethylsilane (TMS) as an internal standard. Chemical shifts (δ) were expressed in ppm with reference to the solvent signals. EI-MS were measured on a VG Auto Spec-3000 spectrometer, and HR-ESI-MS were taken on an API Qstar Pulsar instrument. Column chromatography (CC) was performed on silica gel (200 – 300 mesh, 100 – 200 mesh, 80 – 100 mesh, Qingdao Marine Chemical Factory, Qingdao, China), YMC*GEL ODS-A (12 nm, S-50 μm, Kyoto, Japan), Sephadex LH-20 (Amersham Biosciences AB) and MCI (MCI-gel CHP-20P). Fractions were monitored by TLC, and spots were visualized by heating silica gel plates sprayed with 10 % H_2SO_4 in EtOH. Semipreparative HPLC was performed on an Agilent 1200 liquid chromatograph with Zorbax SB-C_{18} (Agilent, 9.4 mm × 250 mm) column.

Plant material

The fruits of *S. sphenanthera* were purchased in Herb Material Market of Juhuacun, Kunming, Yunnan Province, P. R. China, in July 2005, and were identified by Mrs. Xiao-Lei Li. A voucher specimen (KMUST 2005050101) was deposited at the Laboratory of Phytochemistry, the College of Life Science and Technology, Kunming University of Science and Technology. Dimethy sulfoxide (DMSO), LPS (Escherichia coli 055:B5), Aspirin (ASP) and 3-(4, 5-dimethylthiazol-2-y1)-2, 5-diphenyl-tetrazolium bromide (MTT) were purchased from Sigma Chemical Co. (St. Louis, MO, USA). TNF-α and IL-6 ELISA kits were purchased from Biolegend. Dulbeccos modified Eagle's medium (DMEM) and fetal bovine serum (FBS) were obtained from Invitrogen-Gibco (Grand Island, NY). The purity (> 95 %) of compounds **1** and **7** used for the biological assay *in vitro*, was determined by HPLC.

Extraction and isolation

The air-dried fruits of *S. sphenanthera* (8 kg) were extracted with 70 % aq. Me_2CO (3 × 25 L) at r. t. to yield an extract which was successively extracted with petroleum ether and EtOAc. The EtOAc extract (145 g) was subjected to column chromatography (CC) over silica gel (2 kg, 100 – 200 mesh) eluting with a $CHCl_3/Me_2CO$ gradient system (1 : 0, 20 : 1, 10 : 1, 8 : 2, 7 : 3, 6 : 4, 5 : 5, 0 : 1) to give fractions A–H. Fraction B (22 g, $CHCl_3/Me_2CO$ 20 : 1) also was chromatographed over a silica gel (600 g, 200 – 300 mesh) column, eluting with a petroleum ether/Me_2CO gradient system (20 : 1, 10 : 1, 8 : 2, 7 : 3, 0 : 1), to give fractions B-1 to B-5. Fraction B-2 (8 g) was repeatedly chromatographed over silica gel and RP-18, and then recrystallized to yield compounds **4** (3 mg), **6** (5 mg), **13** (25 mg) and **14** (18 mg). Fraction B-3 (10 g) was purified by Sephadex LH-20, silica gel, and finally its components separated by semipreparative HPLC with 65 – 76 % (flow rate 3.0 mL min^{-1}) MeOH/H_2O to afford compounds **1** (6 mg), **2** (8 mg), **3** (9 mg), **5** (14.5 mg), **7** (20 mg), **8** (80 mg), **9** (21.3 mg), **10** (7.6 mg), **11** (6 mg) and **12** (11 mg).

*Methylgomisin O (**1**):* colorless amorphous powder. – $[\alpha]_D^{27}$ = +9.62 (c = 0.156, $CHCl_3$). – CD (c = 0.05, MeOH): 222 (+32.24), 252 (−20.87). – ^{1}H and ^{13}C NMR data see Table 1. – MS (EI, 70 eV): *m/z* (%) = 430 (93) $[M]^+$, 399 (28), 398 (100), 383 (11), 374 (16), 343 (26), 329 (13), 313 (16), 312 (29), 172(5). – HRMS ((+)-ESI): *m/z* = 453.1881 (calcd. 453.1889 for $C_{24}H_{30}O_7Na^+$, $[M+Na]^+$).

*Chloromethyl schisantherin B (**2**):* colorless oil. – $[\alpha]_D^{27}$ = −29.66 (c = 0.118, CH_3COCH_3). – CD (c = 0.05, MeOH): 226 (+19.20), 256 (−15.52). – ^{1}H and ^{13}C NMR data see Table 1. – MS (EI, 70 eV): *m/z* (%) = 550 (16) $[M+2]^+$, 548 (45) $[M]^+$, 512 (9), 448 (29), 359 (25), 343 (86), 331 (66), 316 (40), 300 (100), 83 (49), 55 (53). – HRMS ((+)-ESI):

m/z = 571.1703 (calcd. 571.1710 for $C_{28}H_{33}O_9ClNa^+$, $[M+Na]^+$). – Anal. for $C_{28}H_{33}O_9Cl \cdot 1/2H_2O$: calcd. C 60.27, H 6.14; found C 59.50, H 6.03.

Cell culture

The RAW264.7 mouse macrophage cell line was obtained from the China Cell Line Bank (Beijing, China). Cells were cultured in DMEM supplemented with 3 mm glutamine, antibiotics (100 U mL^{-1} penicillin and 100 U mL^{-1} streptomycin), and 10 % heat-inactivated fetal bovine serum. The cells were maintained at 37 °C in a humidified incubator containing 5 % CO_2. In all experiments, cells were allowed to acclimate for 24 h before any treatments. Compounds **1** and **7** were always added 1 h prior to LPS treatment.

MTT assay for cell viability

To measure cell viability, the MTT assay was performed. RAW264.7 cells were mechanically scraped, seeded in 96-well plates at 4×10^5 cells mL^{-1}, and incubated in a 37 °C, 5 % CO_2 incubator overnight. After 24 h, the cells were treated with 50 μL aliquots with different concentrations of compounds **1** and **7** or 10 nm ASP (0.5 – 12.5 mg L^{-1}) for 2 h, followed by stimulation with 50 μL of LPS for 18 h. Subsequently, 20 μL of 5 mg mL^{-1} MTT in FBS-free medium was added to each well and incubated for an additional 4 h. Cell-free supernatants were then removed and resolved with 150 μL per well DMSO. The optical density was measured at 570 nm on a microplate reader.

Monitor cytokine in vitro

To investigate the effect of compounds **1** and **7** on cytokine responses from LPS-treated cells, RAW264.7 cells (4×10^5) were seeded into 24-well plates, pretreated with 0.5, 2.5 or 12.5 mg/L of **1** and **7** for 1 h prior to treatment with 1 mg L^{-1} of LPS for 12 h. Cells were treated with 10 nM ASP as a positive control. Cell-free supernatants were collected and stored at −20 °C until assayed for cytokine levels. The concentrations of TNF-α and IL-6 in the cell supernatants were determined using an ELISA kit. Concentrations were determined for six wells of each sample.

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