Two New Sesquiterpenes from *Inula salsoloides* and Their Inhibitory Activities against NO Production

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Two new sesquiterpenes, inulasalene (**1**) and inulasalsolide B (**2**), as well as 22 known compounds were isolated from the aerial parts of *Inula salsoloides*. The structures of the new sesquiterpenes were determined on the basis of their spectroscopic data and chemical properties. Compounds **1**–**4** exhibited potent inhibitory activities against NO production with IC_{50} values range of 0.010–1.290 μg/ml in lipopolysaccharide (LPS)-stimulated RAW264.7 cells.

Introduction. – The genus of *Inula* (Asteraceae) has a broad distribution in the world, and phytochemical studies revealed several types of constituents such as sesquiterpenes, flavonoids, and terpenoids. Sesquiterpenes were predominant within this genus [1][2]. *Inula salsoloides* (TURC.) OSTENF. of this genus broadly distributed in West and North of China. The entire herb before flowering can be used in the treatment of fever and diuresis. Recent phytochemical investigations of this plant led to the isolation of several sesquiterpene lactones [3][4]. As part of our continuing search for bioactive components from Chinese medicinal plants, we studied the aerial parts of *I. salsoloides* grown in Shan′xi province of China and isolated 23 compounds including two new sesquiterpenes. Here, we report the isolation and structure elucidation of two new sesquiterpenes. In addition, the inhibitory activities of all seven isolated sesquiterpenes against lipopolysaccharide (LPS)-induced NO production in RAW264.7 macrophages were also evaluated.

Results and Discussion. – The air-dried, powdered aerial parts of *I. salsoloides* were extracted with 75% EtOH three times at room temperature. The EtOH extract was partitioned with petroleum ether, AcOEt, and BuOH. Part of AcOEt fraction was subjected to various types of column chromatography (SiO_2, *Sephadex LH-20*, and prep. HPLC) to afford 24 compounds, including two new sesquiterpenes, inulasalene (**1**) and inulasalsolide B (**2**), five known sesquiterpenes (**3**–**7**; see *Fig. 1*), seven known flavonoids, and ten other compounds.

Compound **1** was obtained as colorless needles. The molecular formula of **1** was determined as $C_{15}H_{28}O_4$ by HR-ESI-MS (*m*/*z* 273.1200 ($[M+H]^+$, $C_{15}H_{29}O_4^+$; calc. 273.1203). The ^{1}H-NMR spectrum of **1** displayed signals for four Me groups at δ(H) 0.73 (*t*, *J* = 6.0, Me(13)), 0.83 (*t*, *J* = 6.0, Me(12)), 0.84 (*d*, *J* = 7.0, Me(15)), and 1.64 (*s*,

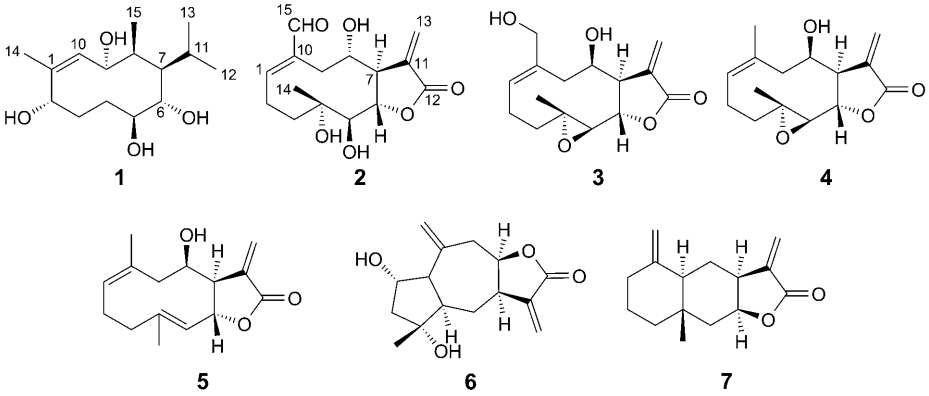

Fig. 1. *Structures of sesquiterpenes* **1**–**7** *from* Inula salsoloides

Me(14)), four OH groups at δ(H) 4.20, 4.31, 4.47, and 4.59 (*s*, each 1 H), and one alkene H-atom at δ(H) 5.31 (*s*). The ^{13}C-NMR and DEPT data indicated 15 C-atoms, including four Me, two CH_2, and eight CH, four of which are O-bearing CH groups, and one quaternary C-atom (*Table 1*). The ^{1}H,^{1}H-COSY spectrum of **1** showed the long chain connectivities from H–C(2) to H–C(7), from H–C(7) to Me(12) and Me(13), and from H–C(7) to H–C(10) (*Fig. 2*). In the HMBC spectrum, correlations HO–C(2)/C(2), HO–C(5)/C(5), HO–C(6)/C(6), and HO–C(9)/C(9) were observed. Furthermore, correlations Me(14)/C(1), C(2), and C(10), and Me(15)/C(7), C(8), and

Table 1. *^{1}H- and ^{13}C-NMR* (500 and 125 MHz, resp.) *Data of Compound* **1** *in (D_6)DMSO.* δ in ppm, *J* in Hz.

	δ(H)	δ(C)		δ(H)	δ(C)
C(1)		135.2	H–C(11)	1.89–1.91 (*m*)	34.1
H–C(2)	3.73–3.76 (*m*)	65.8	Me(12)	0.83 (*t*, *J* = 6.0)	12.0
CH_2(3)	1.21 (*dd*, *J* = 5.0, 4.0), 1.51–1.53 (*m*)	29.8	Me(13)	0.75 (*t*, *J* = 6.0)	16.7
CH_2(4)	1.09–1.11 (*m*), 1.34–1.36 (*m*)	26.2	Me(14)	1.64 (*s*)	20.3
H–C(5)	3.57 (*dd*, *J* = 6.0, 4.0)	70.5	Me(15)	0.84 (*d*, *J* = 7.0)	20.9
H–C(6)	3.35 (br. *s*)	64.6	HO–C(2)	4.59 (*s*)	
H–C(7)	1.09–1.11 (*m*)	48.2	HO–C(5)	4.31 (*s*)	
H–C(8)	1.49–1.52 (*m*)	41.1	HO–C(6)	4.20 (*s*)	
H–C(9)	3.67 (*d*, *J* = 8.0)	67.6	HO–C(9)	4.47 (*s*)	
H–C(10)	5.31 (*s*)	130.1			

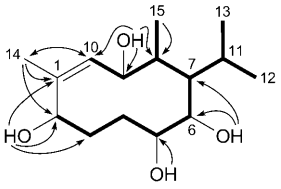

Fig. 2. *Selected ^{1}H,^{1}H-COSY correlations and HMBCs of* **1**

C(9) were also observed. The correlations Me(12)/Me(13) and C(7)/C(11) indicated that an ^{i}Pr group was located at C(7). In the NOESY spectrum, correlations HO–C(6)/H–C(7), Me(15)/H–C(9) and Me(13), and HO–C(5)/Me(12) were observed (*Fig. 3*). Based on these evidences, compound **1** was identified as germacrane sesquiterpene, and named inulasalene.

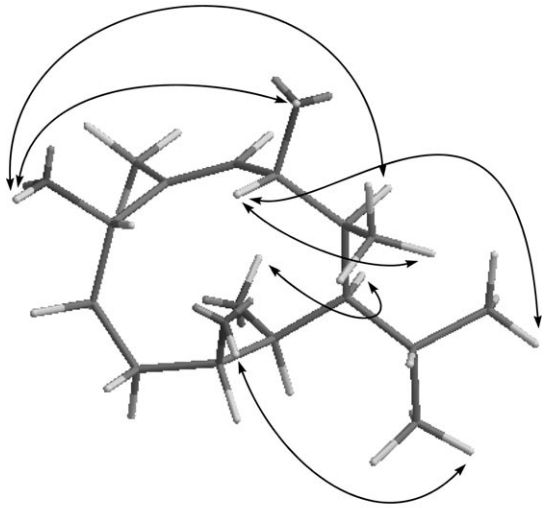

Fig. 3. *Selected NOESY correlations and relative configuration of* **1**

Compound **2** was obtained as colorless needles. The molecular formula of **2** was determined as $C_{15}H_{20}O_6$ on the basis of HR-ESI-MS (*m/z* 295.1180 ($[M-H]^-$, $C_{15}H_{19}O_6^+$; calc. 295.1182). The ^{13}C-NMR and DEPT spectra indicated the presence of 15 C-atoms, including one Me group, four CH_2 and six CH groups, and four quaternary C-atoms, including two CO C-atoms. The ^{1}H- and ^{13}C-NMR data were very similar to those of inulasalsolide (**3**), except for the appearance of the signal corresponding to one aldehyde group and the different chemical shift of C(1) [3]. Resonances of a pair of olefinic C-atoms at δ(C) 137.8 (C(11)) and 121.5 (C(13)), and of exocyclic olefinic H-atoms at δ(H) 5.65 (*d*, $J=3.5$, H_a–C(13)) and 6.25 (*d*, $J=3.5$, H_b–C(13)) indicated the existence of a characteristic α-methylidene-γ-lactone functionality. In the HSQC spectrum of **2**, the signal at δ(H) 9.41 (*s*) correlated with the C-atom signal at δ(C) 197.7 (*Table 2*). In ^{1}H,^{1}H-COSY spectrum, H–C(2) showed two patterns of connections from H–C(1) to H_a–C(3)/H_b–C(3), and from H–C(5) to H–C(9) (*Fig. 4*). In the HMBC spectrum, the correlations Me(14)/C(3), C(4), and C(5); CH_2(13)/C(7), C(11), and C(12); H–C(5)/C(4), C(6), and C(7); and CH_2(9)/C(7), C(8), C(10), and C(15) were observed. The aldehyde group was located at C(1) as evidenced by the HMBCs of H–C(15)/C(1), C(9) and C(10) (*Fig. 4*). The relative configuration was determined on the basis of the coupling constant and NOESY experiment. The large coupling constant of 9.5 Hz between H–C(6) and H–C(7) suggested the *trans*-configuration of the α-methylidene-γ-lactone ring. In the NOESY spectrum, the correlations H–C(8)/H–C(6) and Me(14); and H–C(5)/H–C(7) were also observed

Table 2. *^{1}H- and ^{13}C-NMR* (500 and 125 MHz, resp.) *Data of Compounds* **2** *and* **3** (in CD_3OD, δ in ppm, J in Hz)

	δ(H)		δ(C)	
	2	**3**	**2**	**3**
H–C(1)	6.81 (*dd*, *J* = 6.0, 2.0)	5.56 (*dd*, *J* = 3.5, 3.5)	156.2	132.4
CH_2(2)	2.61–2.63 (*m*), 2.62–2.65 (*m*)	2.20–2.23 (*m*), 2.58–2.61 (*m*)	26.0	25.0
CH_2(3)	1.25–1.27 (*m*), 2.30–2.32 (*m*)	1.35 (*dd*, *J* = 7.0, 6.0), 2.12 (*dd*, *J* = 6.0, 5.0)	36.9	37.1
C(4)			61.1	63.1
H–C(5)	2.84 (*d*, *J* = 9.5)	3.00 (*d*, *J* = 9.0)	64.5	67.5
H–C(6)	4.31 (*dd*, *J* = 9.5, 9.5)	4.48 (*t*, *J* = 8.5, 7.5)	77.3	76.8
H–C(7)	2.75–2.77 (*m*)	3.29 (*dd*, *J* = 7.0, 7.0)	48.2	51.4
H–C(8)	5.14 (*dd*, *J* = 9.0, 8.5)	4.50–4.52 (*m*)	65.5	75.0
CH_2(9)	2.37 (*dd*, *J* = 10.0, 1.5), 2.77–2.80 (*m*)	2.54 (*dd*, *J* = 10.0, 1.0), 2.80 (*dd*, *J* = 4.5, 4.5)	33.4	44.6
C(10)			144.0	136.7
C(11)			137.8	140.1
C(12)			170.1	170.2
CH_2(13)	5.65 (*d*, *J* = 3.5), 6.25 (*d*, *J* = 3.5)	5.58 (*d*, *J* = 3.0), 6.32 (*d*, *J* = 3.0)	121.5	122.6
Me(14)	1.58 (*s*)	1.32 (*s*)	18.2	17.2
H–C(15)	9.41 (*s*)		197.0	
CH_2(15)		4.02 (*d*, *J* = 12.0), 4.39 (*d*, *J* = 11.5)		61.2

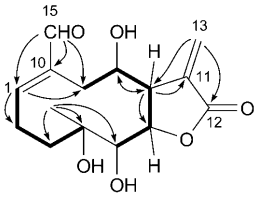

Fig. 4. *Selected ^{1}H,^{1}H-COSY correlations and HMBCs of* **2**

(*Fig. 5*). Based on the above results and comparison with the X-ray diffraction analysis of **3** (*Fig. 6*), **2** was identified and named inulasalsolide B.

The 21 known compounds were identified as inulasalsolide (**3**) [3], 4α,5β-epoxyeupatolide (**4**) [4], eupatolide (**5**) [5], florilenalin (**6**) [6], isoalantolactone (**7**) [7], kaempferol [8], acacetin [9], luteolin [10], 4′,7-dihydroxyflavone [11], 2-(3,5-dihydroxyphenyl)-5,7-dihydroxyflavone [12], apigenin [13], 3′,4′,7-trihydroxy-5-methoxyflavone [14], β-sitosterol, *cis*-1-*p*-menthene-3,6-diol [15], dodecane, *p*-hydroxybenzonic acid, ethyl *p*-hydroxybenzoate [16], stigmasterol [17], physcion [18], 8,9,10-trihydroxythymol [19], and 9,10-dihydroxythymol [20], by comparison of their spectroscopic data with literature values.

In the present study, compounds **1** and **2**, together with the other five known sesquiterpenes **3**–**7** isolated from *I. salsoloides*, were evaluated for their inhibitory activities on NO production induced by LPS in RAW264.7 macrophages (*Table 3*). New sesquiterpenes **1** and **2** inhibited the NO production with IC_{50} values of 1.290 and 0.270 μg/ml, respectively. Sesquiterpenes **3** and **4** with the epoxy ring at C(4) and C(5)

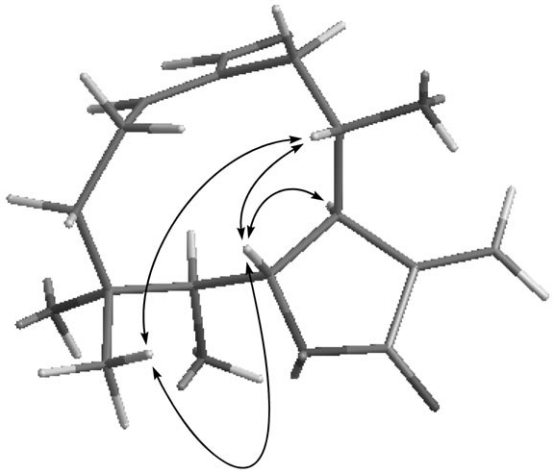

Fig. 5. *Selected NOESY correlations and relative configuration of* **2**

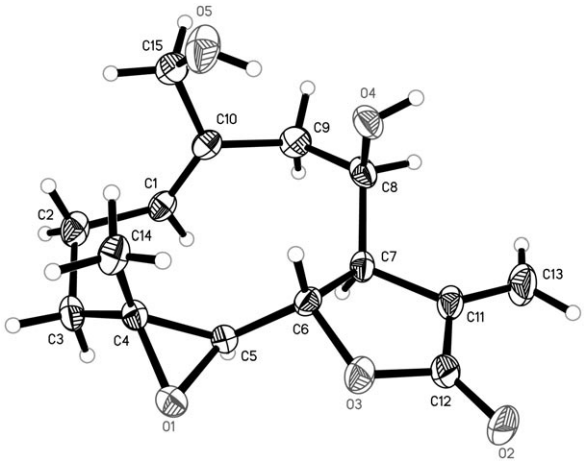

Fig. 6. *Single-crystal X-ray structure of* **3**

showed potent inhibitory activities with IC_{50} values of 0.013 and 0.010 μg/ml, implying that the epoxy ring enhances the inhibitory activity.

Experimental Part

General. Column chromatography (CC): *Sephadex LH-20* (*GE Healthcare Bio-science AB*, Sweden) and silica gel (SiO_2; 100–200 and 200–300 mesh; *Yantai Jiangyou*, P. R. China). Semi-prep. HPLC: *Shimadzu LC-6AD* series equipped with an *SPD-20* spectrophotometer using a *ZORBAX SB-C_{18}* column, 5 μm (9.4 × 250 nm, i.d.). TLC: *HSG F_{254}* silica-gel plates (10–40 μm; *Yantai Huiyou*, P. R. China). NMR Spectra: *Bruker DRX-500* spectrometer (^{1}H: 500 MHz and ^{13}C: 125 MHz), with TMS as an internal standard. EI-MS: *Autospec-UltimaETOF* apparatus. ESI-MS: *Agilent 1100 series* mass spectrometer. HR-ESI-MS: *Q-TOF micro YA019* mass spectrometer.

Table 3. IC_{50} *Values* [μg/ml] *of Isolated Sesquiterpenes* **1**–**7** *against NO Production in LPS-Stimulated RAW264.7 Cells*

Compounds	IC_{50} [μg/ml]	Compounds	IC_{50} [μg/ml]
1	1.290	**5**	0.338
2	0.270	**6**	0.192
3	0.013	**7**	0.231
4	0.010	AG[a])	114.900

[a]) AG = Aminoguanidine.

Plant Material. Aerial parts of *I. salsoloides* were collected in Dingbian, Shan'xi Province in P. R. China, in August, 2007. The plant material was identified by Prof. *B. Huang* and *H. Zheng*, Department of Phytochemistry, Second Military Medical University.

Extraction and Isolation. The air-dried, powdered aerial parts of *I. salsoloides* (18.0 kg) were extracted three times with 75% EtOH (3 × 20 l, each 24 h) at r.t. The extract was evaporated *in vacuo* to leave a residue (1010.0 g), which was taken up in H_2O and extracted in succession with petroleum ether (PE), AcOEt, and BuOH, yielding 345.5, 146.5, and 124.8 g of extracts, resp. Part of AcOEt fraction (100.0 g) was subjected to CC (SiO_2 (100–200 mesh, 10 × 60 cm); stepwise gradient of CH_2Cl_2/MeOH 100:1, 50:1, 20:1, 10:1, 5:1 and 1:1, each 15 l) to afford ten fractions, *Frs. 1–10*. *Fr. 5* were purified by CC (SiO_2 (200–300 mesh, 5 × 50 cm); CH_2Cl_2/MeOH gradient; and *Sephadex LH-20* CC (1 × 100 cm); CH_2Cl_2/MeOH 1:1) to give compound **1** (25 mg), kaempferol (12 mg), luteolin (9 mg), and apigenin (7 mg). *Fr. 4* was purified by CC (SiO_2 (200–300 mesh, 5 × 50 cm); CH_2Cl_2/MeOH gradient), and four fractions were obtained, *Frs. 4A–4D*. *Fr. 4B* was further separated by CC (*Sephadex LH-20*; CH_2Cl_2/MeOH 1:1) to give compounds **2** (54 mg) and **3** (5 mg). Compounds **4** (10 mg) and **7** (11 mg) were obtained from the *Fr. 4C* by CC (SiO_2; 200–300 mesh, 1 × 30 cm) and recrystallized (CH_2Cl_2/MeOH/acetone 1:1:1), resp. Compounds β-sitosterol (12 mg), *cis*-1-*p*-menthene-3,6-diol (15 mg), dodecane (20 mg), *p*-hydroxybenzoic acid (9 mg), and ethyl *p*-hydroxybenzoate (20 mg) were isolated from *Fr. 1* by repeated CC (SiO_2, 200–300 mesh, 3 × 50 cm). Compounds 4′,7-dihydroxyflavone (10 mg), 8,9,10-trihydroxythymol (5 mg), and 9,10-dihydroxythymol (3 mg) were obtained from *Fr. 2* by CC (SiO_2, 200–300 mesh, 3 × 50 cm). Compounds **5** (7 mg) and **6** (5 mg), as well as acacetin (11 mg), 2-(3,5-dihydroxyphenyl)-5,7-dihydroxyflavone (11 mg), 3′,4′,7-trihydroxy-5-methoxyflavone (15 mg), physcion (6 mg), and stigmasterol (20 mg) were isolated from *Fr. 6* by CC (SiO_2, 200–300 mesh, 3 × 50 cm; and *Sephadex LH-20* CC; CH_2Cl_2/MeOH 1:1).

Inulasalene (= rel-*(1S,2S,3R,4S,5S,6Z,8S)-4,7-Dimethyl-3-(1-methylethyl)cyclodec-6-ene-1,2,5,8-tetrol*; **1**): Colorless needles. M.p. 156–157°. $[\alpha]_D^{25} = -285$ ($c = 0.05$, $CHCl_3$). ^{1}H- and ^{13}C-NMR: see *Table 1*. ESI-MS: 273.4 ($[M+H]^+$). HR-ESI-MS: 273.1200 ($[M+H]^+$, $C_{15}H_{29}O_4^+$; calc. 273.1203).

Inulasalsolide B (= rel-*(3aR,4S,6E,11R,11aS)-2,3,3a,4,5,8,9,10,11,11a-Decahydro-4,10,11-trihydroxy-10-methyl-3-methylidene-2-oxocyclodeca[b]furan-6-carbaldehyde*; **2**): Colorless needles. M.p. 173–174°. $[\alpha]_D^{25} = -131$ ($c = 0.09$, $CHCl_3$). ^{1}H- and ^{13}C-NMR: see *Table 2*. ESI-MS: 295.3 ($[M-H]^-$). HR-ESI-MS: 295.1180 ($[M-H]^-$, $C_{15}H_{19}O_6^-$; calc. 295.1182).

Evaluation of Nitric Oxide (*NO*) *Production in Lipopolysaccharide* (LPS)*-Stimulated RAW264.7 Cells.* RAW264.7 Macrophages were seeded into 24-well cell-culture plates (1×10^5 cells per well). The cells were co-incubated with tested compounds and LPS (1 μg/ml) for 24 h. The amount of NO was assessed by determining the nitrite concentration in the culture supernatants with *Griess* reagent. Aliquots of supernatants (100 μl) were incubated in sequence with 50 μl 1% sulphanilamide and 50 μl 0.1% *N*-(1-naphthyl)ethylenediamine in 2.5% phosphoric acid soln. The absorbance at 570 nm was read using a microplate reader.

Cytotoxicity Assay. Cytotoxicity assay was carried out 24 h after cells were seeded according to the protocol reported by *Denizot* and *Lang* [21]. Briefly, different concentrations of the tested compounds (*Fig. 1*; purity > 98% by HPLC) were added, and incubation was continued for 72 h. Cell viability was

evaluated by measuring the optical density of the color produced by MTT (= 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2*H*-tetrazolium bromide) dye reduction with a microplate reader at 570 nm.

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