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Inhibitors of the LPS-induced NF-κB activation from *Artemisia sylvatica*

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Abstract

Three guaianolide sesquiterpene lactones, 3α,4α-epoxyrupicolins C–E, together with six known sesquiterpenes, artemisolide, 3-methoxytanapartholide, deacetyllaurenobiolide, moxartenolide as well as arteminolides B and D were isolated by bioassay-guided fractionation from the methanol extract of the aerial parts of *Artemisia sylvatica* using the NF-κB mediated reporter gene assay. All isolated compounds displayed inhibitory activity on the LPS-induced NF-κB activation, NO production, and TNF-α production with IC_{50} values of 0.49–7.17, 1.46–6.16, and 3.19–27.76 μM, respectively, in RAW264.7 cells. It was also established that arteminolide B suppressed the expression of NF-κB target genes such as iNOS and COX-2. This is the first report of NF-κB inhibitory activities of these compounds and supports the pharmacological use of *Artemisia sylvatica*, which has been employed as an herbal medicine for the treatment of inflammation.

Keywords: *Artemisia sylvatica*; Compositae; Sesquiterpene lactone; NF-κB; NO; TNF-α; iNOS; COX-2

1. Introduction

The transcriptional activators of the nuclear factor-κB (NF-κB)/Rel family have a central role in the expression of a number of genes encoding cytokines such as TNF-α, adhesion molecules, and inflammatory enzymes such as inducible nitric oxide synthase (iNOS) and cyclooxygenase-II (COX-2) (Siebenlist et al., 1994; Duffey et al., 1999; De Martin et al., 1999). The activation of NF-κB has been shown to mediate inflammation and suppress apoptosis. Activated NF-κB has been found in various inflammatory diseases, including rheumatoid arthritis, septic shock, and myocardial ischemia (Miagkov et al., 1998; Nichols et al., 2001). Recent works have demonstrated that specific inhibitors of NF-κB might be interesting chemical leads to develop effective therapeutic agents for the treatment of inflammation and cancer (Yamamoto and Gaynor, 2001).

Members of the *Artemisia* genus (Compositae) are important medicinal plants throughout the world. *Artemisia sylvatica* and several related species have been used in Chinese natural medicine, which are prescribed as both hemostatic and sedative agent in Chinese traditional preparations (Yoshikawa et al., 1996). Especially, artemisinin is also well known as an antimalarial agent, which was isolated from *A. annua* (Klayman, 1985). Recently, it has been reported that some sesquiterpene lactones isolated from *A. sylvatica* are inhibitors of farnesyl protein transferase (Lee et al., 1998, 2000). In our search for inhibitors of NF-κB activation from natural sources, the methanol extract of the aerial parts of *A. sylvatica* Maxim. showed a potent inhibition of NF-κB activation

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with IC_{50} value of 5.94 μg/ml in murine macrophage RAW264.7 cells transfected with NF-κB-mediated reporter gene construct (Koo et al., 2001). Bioassay-guided fractionation led to isolation of three new sesquiterpene lactones, 3α,4α-epoxyrupicolins C–E (**1**–**3**) and six known compounds, artemisolide (**4**), 3-methoxytanapartholide (**5**), deacetyllaurenobiolide (**6**), moxartenolide (**7**), and arteminolides B and D (**8** and **9**). Here, we describe their isolation and structure elucidation of **1**–**3**, as well as the biological evaluation of **1**–**9**.

2. Results and discussion

2.1. Structure elucidation of the three new sesquiterpene lactones

Compound **1** was isolated as a white amorphous powder and shown to possess a molecular formula of $C_{20}H_{24}O_6$. (HRESIMS $[M+H]^+$, *m/z* 361.1669). An analysis of its ^1H and ^{13}C NMR spectral data (Table 1) suggested that compound **1** is a guaianolide (Ahmed et al., 2002; Bohlmann et al., 1981; Todorova et al., 1998). The ^1H NMR spectrum of **1** exhibited signals for an angeloyl group at δ_H 6.28 (*d*, *J* = 7.2 Hz, H-3′), 2.05 (*dd*, *J* = 1.5, 7.2 Hz, H-4′), and 1.94 (*s*, H-5′) (Jakupovic et al., 1988), exomethylene protons at δ_H 5.58 (*s*, H-14a) and 5.05 (*s*, H-14b), an α-methylene proton signals of γ-lactone moiety at δ_H 6.24 and 5.65 (both *d*, *J* = 3.0 Hz, H-13a and H-13b), as well as three methine protons at δ_H 3.98 (*dd*, *J* = 9.6, 11.0 Hz, H-6), δ_H 3.33 (*m*, H-7), and δ_H 5.08 (*m*, H-8). Identification of the protons attached to specific carbons was achieved by analysis of the HMQC and DEPT spectrum, which revealed 20 carbon signals in the ^{13}C NMR spectrum for three methyls, four methylenes, six methines, and seven quaternary carbons, including three nonprotonated olefinics and two carbonyls. These results indicated that compound **1** is 3α,4α-epoxyrupicolin B with an angeloyl group attached (Todorova et al., 1998), whose position was determined by HMBC analysis. That is, the proton signal at δ_H 5.08 (*m*, H-8) showed a long-range correlation with the angeloyl carbonyl carbon signal at δ_C 166.83, indicating that the angeloyl group was located at C-8.

The relative configuration was determined by NOESY NMR spectroscopic experiment and the comparison of chemical shift data and coupling constants with the literature (Bohlmann et al., 1981; Todorova et al., 1998). The important NOE correlations were H-3/CH_3-15, H-5/OH-1, H-5/H-7, H-6/H-8, and H-6/CH_3-15. Based on the above-mentioned evidence, 3α,4α-

Table 1
^1H and ^{13}C NMR spectral data for compounds **1**–**3** ($CDCl_3$)[a]

No.	δ_C			δ_H		
	1	**2**	**3**	**1**	**2**	**3**
1	81.89	82.02	82.02			
2a	40.67	40.80	40.79	2.26 *d* (15.0)	2.23 *d* (13.0)	2.22 *d* (13.0)
2b				1.96 *d* (15.0)	1.94 *d* (13.0)	1.94 *d* (13.0)
3	64.32	64.50	64.50	3.61 *s*	3.59 *s*	3.59 *s*
4	67.39	67.62	67.59			
5	61.10	61.26	61.25	2.39 *d* (11.0)	2.37 *d* (10.4)	2.36 *d* (10.4)
6	75.26	75.47	75.47	3.98 *dd* (9.6, 11.0)	3.94 *dd* (9.6, 10.4)	3.94 *dd* (9.6, 10.4)
7	46.87	46.66	46.66	3.33 *m*	3.26 *m*	3.26 *m*
8	72.83	73.18	73.06	5.08 *m*	4.98 *m*	4.98 *m*
9a	35.15	35.48	35.41	2.65 *dd* (2.4, 15.0)	2.59 *dd* (3.0, 13.2)	2.59 *dd* (3.0, 13.2)
9b				2.30 *dd* (6.0, 15.0)	2.28 *dd* (6.0, 13.2)	2.28 *dd* (6.0, 13.2)
10	140.73	140.92	140.91			
11	136.59	136.88	136.79			
12	168.61	168.78	168.74			
13a	122.59	122.76	122.72	6.24 *d* (3.0)	6.24 *d* (3.6)	6.24 *d* (3.0)
13b				5.65 *d* (3.0)	5.64 *d* (3.6)	5.64 *d* (3.0)
14a	117.94	118.23	118.22	5.58 *s*	5.56 *br* s	5.56 *br* s
14b				5.05 *s*	5.01 *br s*	5.01 *br s*
15	18.53	18.80	18.80	1.71 *s*	1.67 *s*	1.67 *s*
1′	166.83	176.02	172.56			
2′	125.99	41.73	43.79		2.45 *m*	2.29 (2H) *bd* (7.2)
3′a	140.49	26.64	25.86	6.28 *d* (7.5)	1.77 *m*	2.18 *m*
3′b					1.52 *m*	
4′	15.91	12.04	22.65	2.05 *dd* (1.5, 7.2)	0.96 *t* (6.0)	1.02 *d* (6.6)
5′	20.44	16.93	22.67	1.94 *s*	1.23 *d* (6.0)	1.01 *d* (6.6)
1-OH				4.08 *s*	4.06 *s*	4.05 *s*

[a] TMS was used as the internal standard; chemical shifts are shown in the δ scale with *J* values (Hz) in parentheses.

epoxyrupicolin C (**1**), was determined as 8-angeloyloxy-1α-hydroxy-3α,4α-epoxy-5α,7αH-10(14),11(13)-guaiadien-12,6α-olide.

The molecular formula of compound **2** was determined as $C_{20}H_{26}O_6$ by HRFABMS ([M + Na]+, *m/z* 385.1624). The ^1H and ^{13}C NMR spectra of **2** were comparable to those of 3α,4α-epoxyrupicolin C (**1**) except for the presence of a 2-methylbutyryl group at δ_H 2.45 (*m*, H-2′), 1.77 (*m*, H-3′a), 1.52 (*m*, H-3′b), 0.96 (*t*, *J* = 6.0 Hz, H-4′), and 1.23 (*d*, *J* = 6.0 Hz, H-5′) instead of the angeloyl group of **1** (Table 1). The HMBC correlation between the proton signal at δ_H 4.98 (*m*, H-8) and the carbonyl carbon signal at δ_C 176.02 demonstrated that the methylbutyryl group was attached to C-8. Finally, the stereochemistry of **2** was determined by a manner similar to that of **1**. Therefore, 3α,4α-epoxyrupicolin D was elucidated as 8α-methybutyryloxy-1α-hydroxy-3α,4α-epoxy-5α,7αH-10(14),11(13)-guaiadien-12,6α-olide.

Compound **3** had the same molecular formula of $C_{20}H_{26}O_6$, as that of **2** by HRFABMS. The ^1H and ^{13}C NMR spectra of **3** were almost superimposable to those of **2** except for the 2-methylbutyryl moiety of the latter compound. The presence of an isovaleryl proton signals at δ_H 2.29 (2H, *bd*, *J* = 7.2 Hz, H-2′), 2.18 (*m*, H-3′), 1.02 (*d*, *J* = 6.6 Hz, H-4′), and 1.01 (*d*, *J* = 6.6 Hz, H-5′) indicated that **3** has an isovaleryl moiety rather than a 2-methylbutyryl group as in **2**. Based on the spectral analysis and reported data, 3α,4α-epoxyrupicolin E (**3**), was elucidated as 8α-sovaleryloxy-1α-hydroxy-3α,4α-epoxy-5α,7αH-10(14),11(13)-guaiadien-12,6α-olide. Additionally, six active compounds of previously known structures were identified as artemisolide (**4**) (Kim et al., 2002), 3-methoxytanapartholide (**5**) (Öksüz, 1990), deacetyllaurenobiolide (**6**) (Quijano et al., 1984), moxartenolide (**7**) (Yoshikawa et al., 1996), arteminolides B and D (**8** and **9**) (Lee et al., 2002a) (Fig. 1).

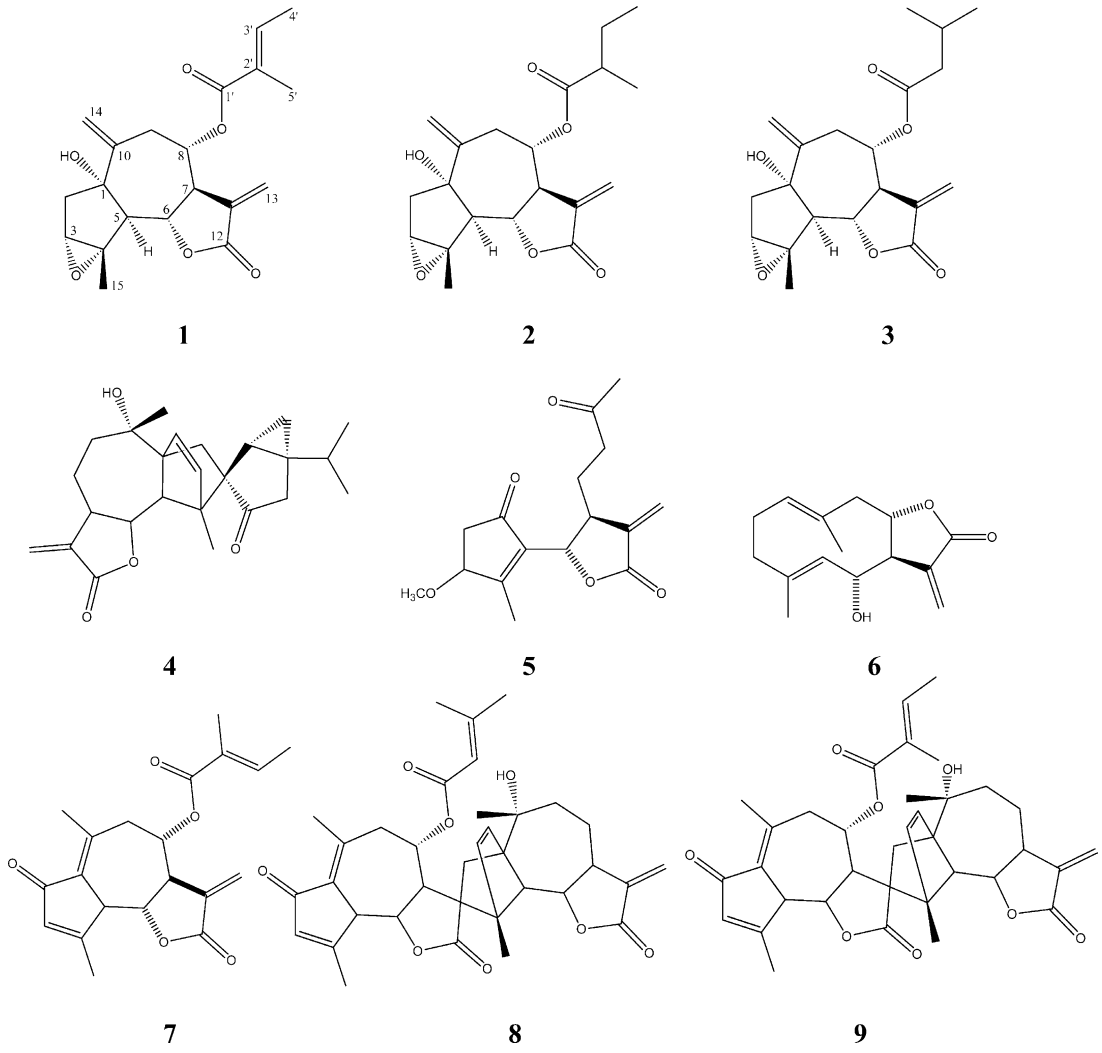

Fig. 1. Chemical constituents from *Artemisia sylvatica*.

2.2. Effects of compounds 1–9 on the NF-κB activation and expressions of NF-κB target genes

Compounds **1**–**9** were examined for their effects on the LPS-induced NF-κB activation using the NF-κB mediated reporter gene assay system (Koo et al., 2001). It was observed that all compounds significantly inhibited LPS-induced NF-κB activation (Table 2). A well-known NF-κB inhibitor, parthenolide, as a standard compound, inhibited NF-κB activation with IC_{50} value of 3.42 μM. Among them, dimeric sesquiterpene lactones, **8** and **9** showed potent inhibitory activity on LPS-induced NF-κB activation with IC_{50} values of 0.49 μM and 0.54 μM, respectively. The new guaianolide, 3α,4α-epoxyrupicolin C (**1**) showed strong inhibitory effect on the NF-κB activation with IC_{50} value of 0.89 μM. The relative potency of these compounds was arteminolide B (**8**) and D (**9**)> artemisolide (**4**) and 3α,4α-epoxyrupicolin C (**1**)>moxartenolide (**7**) > 3α,4α-epoxyrupicolin D (**2**) and 3α,4α-epoxyrupicolin E (**3**) > 3-methoxytanapartholide (**5**) and deacetyllaurenobiolide (**6**) and that of well known parthenolide was comparable to 3 α,4 α-epoxyrupicolin D (**2**) and 3α,4α-epoxyrupicolin E (**3**).

Since NF-κB is an important transcription factor involved in the regulation of the expression of inflammatory NF-κB target genes such as TNF-α, iNOS, and COX-2 (Wong and Menendez, 1999; Pahl, 1999), we determined whether compounds **1**–**9** inhibit the expressions of these genes in LPS-stimulated RAW264.7 cells. Firstly, we examined the effects of compounds **1**–**9** on the LPS-induced production of NO and TNF-α in RAW264.7 cells (Table 2). The cells were pretreated with various concentrations of compounds for 30 min, and subsequently stimulated with 1 μg/ml of LPS, and then the amount of NO and TNF-α in the culture supernatants was determined (see Experimental Section). All compounds significantly inhibited the LPS-induced production of NO and TNF-α in RAW264.7 cells. Consistent with the NF-κB inhibitory activities, compounds **8** and **9** showed potent inhibitory activity on the production of NO and TNF-α. The control, parthenolide, also significantly inhibited the production of NO and TNF-α with IC_{50} values of 2.41 and 2.68 μM, respectively. Cell viability, as measured by the MTT assay, also showed that all compounds had no significant cytotoxicity to the RAW264.7 cells at their effective concentrations for the inhibition of NF-κB activation, NO and TNF-α production (data not shown).

To further confirm that these compounds inhibit LPS-induced NF-κB activation in RAW264.7 cells, we investigated whether these compounds suppress the LPS-induced expressions of iNOS and COX-2 by Western blot analysis. We selected arteminolide B (**8**) since this sesquiterpene showed the most potent inhibitory activity on the LPS induced NF-κB activation, NO production, and TNF-α production among the isolated compounds. As shown in Fig. 2, arteminolide B (**8**) dose-dependently suppressed the LPS-induced expressions of iNOS and COX-2 proteins.

Several studies have investigated the functional Michael acceptors of some sesquiterpenes and diterpenes (Hehner et al., 1999; Kwok et al., 2001; Lyß et al., 1998; Lee et al., 2002b), such as an exomethylene group conjugated to a carbonyl group, which can react with biological nucleophiles, especially the sulfhydryl group of the cysteine residue in the proteins, which play an important role in the NF-κB activation and DNA binding process (Rungeler et al., 1999). All sesquiterpenes **1**–**9** contain a α-methylene-γ-lactone ring as a common functional group. **8** and **9** have three more possible Michael acceptors other than α-methylene-γ-lactone ring, suggesting that the more Michael acceptors in the structure, the stronger to the NF-κB inhibitory activity. Furthermore, among new 3α,4α-epoxyrupicolins C-E (**1**–**3**), **1** showed the most potent effect, suggesting that the angeloyloxy group at C-8 may contribute to the NF-κB inhibition.

In summary, we isolated nine sesquiterpene lactones from *A. sylvatica*. They all inhibited the LPS-induced

Table 2
IC_{50} values (μM)[a] of compounds **1**–**9** on the NF-κB activation, NO production, and TNF-α production

Compound	NF-κB activation	NO production	TNF-α production
1	0.89±0.02	2.34±0.05	7.58±0.22
2	2.73±0.01	6.16±0.12	9.86±0.31
3	2.68±0.06	5.52±0.15	8.86±0.70
4	0.70±0.02	1.96±0.06	7.42±0.11
5	5.89±0.14	5.68±0.16	15.78±0.56
6	7.17±0.16	5.76±0.28	27.76±1.76
7	1.20±0.05	4.82±0.16	8.26±0.26
8	0.49±0.03	1.46±0.05	3.19±0.01
9	0.54±0.02	1.64±0.02	3.47±0.53
PTN	3.42±0.08	2.41±0.06	2.68±0.11
AG		34.18±0.98	

[a] Data are mean±SD from three separate experiments. PTN: parthenolide. AG:aminoguanidine.

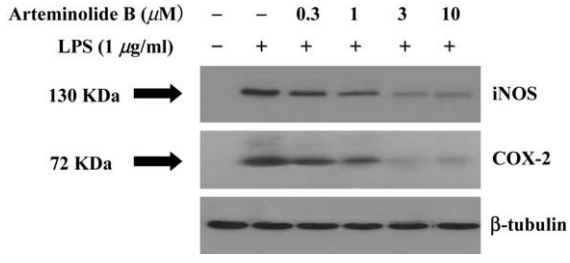

Fig. 2. Effects of arteminolide B on the LPS-induced iNOS and COX-2 expressions. RAW264.7 cells were incubated with or without LPS (1 μg/ml) in the presence of the indicated concentrations of arteminolide B. After 18 h incubation, whole cell lysates were prepared, and Western blot analysis was carried out with antibodies specific for iNOS and COX-2 as described in Experimental section.

NF-κB activation and the expressions of NF-κB target genes. These results may provide a scientific support for the pharmacological basis of *A. sylvatica*, which has been used as an herbal medicine for the treatment of inflammation. However, the mechanism of these sesquiterpenes on the inhibition of NF-κB activation was still unclear. Further studies remain to be elucidated how arteminolide B regulates the NF-κB activation.

3. Experimental

3.1. General

Melting points were measured on an Electrothermal 9100 instrument without correction. Optical rotation $[\alpha]_D^{25}$: JASCO DIP-370 polarimeter. UV: UV-1601 UV–Vis spectrophotometer (Shimadzu). IR: Jasco Report-100 IR spectrometer. NMR: Bruker DMX 600 NMR spectrometer with $CDCl_3$ as a solvent. HRFABMS and HRESIMS: JMS-HX110A/HX110A Tandem Mass spectrometer (JEOL) and Mariner^{TM} Biospectrometry Workstation (ABI, USA), respectively. Preparative HPLC: Spectraphysics SP8800 (USA). Fetal bovine serum, media and supplement materials for cell culture: GIBCO-BRL (Gaithersberg, MD, USA).

3.2. Plant material

The aerial parts of *Artemisia sylvatica* were collected at Ganghwa, Gyeonggi, Korea, in June 2002 and identified by Professor Y. H. Kim, School of Pharmacy, Chungnam National University. A voucher specimen (No. 020908) has been deposited in the Korea Research Institute of Bioscience and Biotechnology.

3.3. Extraction and isolation

The dried aerial parts of *A. sylvatica* (12 kg) were extracted with MeOH at room temperature. The MeOH extract (1.76 kg) was partitioned between H_2O and hexane to obtain a hexane-soluble extract (185 g). The hexane fraction (50 g), exhibiting potent inhibitory effect on the NF-κB activation, was applied to a Si gel column (6×40 cm) eluting with a step gradient of CH_2Cl_2-MeOH (100:0, 50:1, 20:1, 10:1, 5:1, 1:1, each 3 L) to give 12 fractions (Fr1–Fr12). Active Fr3 and Fr4 were combined and subjected to a Si gel column chromatography with mixtures of hexane–EtOAc (20:1, 10:1, 5:1, 2:1, 1:1, EtOAc) as eluents in a stepwise gradient mode to obtain 10 fractions (Fr34-1–Fr34-10). Subfraction 34-5 (830 mg) and subfraction 34-6 (430 mg) were combined and subjected to preparative HPLC (ODS-H80, 150×20 mm, YMC, Japan, CH_3CN-H_2O, 30:70) to afford compound **1** (14.2 mg), compound **2** (5.0 mg) and compound **3** (4.1 mg).

Subfraction 34-7 was subjected to Si gel column chromatography with isocratic solvent mixture hexane–EtOAc (5:1) to give eight fractions (Fr34-7-1–Fr34-7-8). The active fraction 34-7-4 was subjected to preparative HPLC (CH_3CN–H_2O, 15:85) to obtain compounds **8** (4.2 mg) and **9** (3.9 mg). Active subfractions 34-7-5 and 6 were subjected to preparative HPLC (CH_3CN–H_2O, 15:85) to afford compound **7** (17.8 mg). Active Fr34-7-8 was subjected to preparative HPLC (CH_3CN–H_2O, 40:60) to afford compound **4** (21.6 mg).

The Fr34-8 and 34-9 (1.88 g) were combined and subjected to Si gel column chromatography eluted with CH_2Cl_2–MeOH (50:1, 20:1, 10:1, 5:1) to give seven fractions (Fr34-89-1–Fr34-89-7). Subfraction 34-89-2 was subjected to preparative HPLC (CH_3CN–H_2O, 10:90) to obtain compounds **5** (16.2 mg) and **6** (11.7 mg).

3.3.1. 3α,4α-Epoxyrupicolin C (*1*)

White amorphous powder; mp 88–89 °C; $[\alpha]_D^{25}$ −5.83° (*c* 0.03, MeOH); UV (MeOH) λ_{max} (log ε) 202 (4.63) nm; IR (KBr) ν_{max} 3470, 2926, 1760, 1715, 1640, 1140 cm^{-1}; For ^1H and ^{13}C NMR spectral data, see Table 1; HMBC (600 MHz, $CDCl_3$) [C-1, OH-1, H-2ab, H-9a, H-14ab], [C-3, H-5, Me-15], [C-4, H-2b, H-6, Me-15], [C-7,H-9 a,H-13 ab], [C-10, OH-1, H-2a, H-8], [C-12, H-7, H-13ab], [C-14, H-9a], [C-1′, H-8, H-3′ H-4′, H-5′]; NOESY (600 MHz, $CDCl_3$) [H-3, Me-15], [H-5, H-7], [H-5, Me-15], [H-5, OH-1], [H-6, H-8], [H-6, Me-15]; ESI-MS *m/z* 383.4 $[M+Na]^+$, 359.2 $[M+H]^+$; HRESIMS *m/z* 361.1669 $[M-H]^+$ (calcd for $C_{20}H_{25}O_6$, 361.1651).

3.3.2. 3α,4α-Epoxyrupicolin D (*2*)

White needles; mp 118–119 °C; $[\alpha]_D^{25}$ −10.22° (*c* 0.01, MeOH); UV (MeOH) λ_{max} (log ε) 200 (4.34) nm; IR (KBr) ν_{max} 3460, 2965, 1760, 1732 cm^{-1}; For ^1H and ^{13}C NMR spectral data, see Table 1; HMBC (600 MHz, CDCl3) [C-1, OH-1, H-2ab, H-9a, H-14ab], [C-3, H-5, Me-15], [C-4, H-2b, H-6, Me-15], [C-7, H-5, H-9ab, H-13ab], [C-10, OH-1, H-2a, H-8, H-9a], [C-12, H-13ab], [C-14, OH-1, H-9ab], [C-1′, H-8, H-2′, H-3′ab, H-5′]; NOESY (600 MHz, $CDCl_3$) [H-3, Me-15], [H-5, Me-15], [H-5, OH-1], [H-6, Me-15], [H-14a, OH-1]; ESI-MS *m/z* 385.4 $[M+Na]^+$, 361.2 $[M-H]^{+;}$ HRFABMS *m/z* 385.1624 $[M+Na]^+$ (calcd for $C_{20}H_{26}O_6Na$, 385.1627).

3.3.3. 3α,4α-Epoxyrupicolin E (*3*)

White needles; mp 117–118 °C $[\alpha]_D^{25}$ −21.55° (*c* 0.01, MeOH); UV (MeOH) λ_{max} (log ε) 200 (4.34) nm; IR (KBr) ν_{max} 3460, 2965, 1760, 1740 cm^{-1}; For ^1H and ^{13}C NMR spectral data, see Table 1; HMBC (600 MHz, $CDCl_3$) [C-1, OH-1, H-2ab, H-9a, H-14ab], [C-3, H-5, Me-15], [C-4, H-2b, H-6, Me-15], [C-7, H-5, H-9a, H-13ab], [C-9, OH-1, H-14ab], [C-10, OH-1, H-2a, H-8, H-14a], [C-12, H-13ab], [C-14, OH-1, H-9ab],

[C-1′, H-8, H-2′, H-3′]; NOESY (600 MHz, $CDCl_3$) [H-3, Me-15], [H-5, Me-15], [H-5, OH-1], [H-6, H-8], [H-6, Me-15], [H-14a, OH-1]; ESI-MS *m/z* 385.4 $[M+Na]^+$, 361.2 $[M-H]^+$;HRFABMS *m/z* 385.1624 $[M+Na]^+$ (calcd for $C_{20}H_{26}O_6Na$, 385.1627).

3.4. Biological activity

3.4.1. Measurement of NF-κB activity

NF-κB activity was determined as previously described (Koo et al., 2001). RAW264.7 cells stably transfected with a plasmid containing 8 copies of κ elements linked to SEAP (secreted alkaline phosphatase) gene were used.

3.4.2. Measurement of nitric oxide

RAW264.7 cells were seeded in 96 well plates at 1×10^5 cells/well. After 3 h, the cells were treated with various concentrations of compounds and stimulated for 24 h with or without 1 μg/ml of LPS (Sigma Chemical Co., St. Louis, MO, USA). As a parameter of NO synthesis, nitrite concentration was measured in the supernatant of RAW264.7 cells by the Griess reaction as previously described (Schmidt and Kelm, 1996; Jin et al., 2002).

3.4.3. Measurement of TNF-α

TNF-α production was quantitated using Opt-ELATM assay kit according to the manufacture's instructions (Pharmingen, San Diego, USA). Briefly, RAW264.7 cells were seeded in the 96 well plates at a density of 1×10^4 cells/well, pretreated with different concentrations of compounds 1 h, and then the cells were stimulated with LPS (1 μg/ml for 18 h). The wells of the immuno-plate were incubated overnight at 4 °C with 100 μl anti-mouse TNF-α monoclonal antibody. Before performing the next assay steps, the coated plates were washed with washing buffer (PBS with 0.05% Tween 20) and blocked by 200 μl assay diluent (PBS with 10% FBS, pH 7.0) for 1 h. Recombinant mouse TNF-α was diluted and used as a standard. All reagents used in this assay were incubated for 2 h. Assay plates were washed and then exposed to 100 μl working detector (Detection Antibody-Biotinylated mouse TNF-α monoclonal antibody and Enzyme Reagent-Avidin-horseradish peroxidase conjugate) for 1 h. Then the plates were washed again and added 100 μl substrate solution [hydrogen peroxide and 3,3′,5,5′-tetramethylbenzidine (TMB)] at room temperature in dark. After 30 min, the reaction was terminated by adding 50 μl stop solution (2N H_2SO_4), with the absorbance recorded at 450 nm using a microplate reader.

3.4.4. Western blot analysis

RAW264.7 cells (60 mm dish) were incubated with various concentrations of tested compounds and 1 μg/ml of LPS for 18 h. The cells were washed with ice-cold PBS three times, lysed, and homogenized in 50 μl of ice-cold lysis buffer (50 mM Tris–HCl, pH 7.5, 1% Nonidet P-40, 1 mM EDTA, 1 mM phenylmethyl sulfonyl fluoride, 1 mM Na_3VO_4, 50 mM NaF, 1 μg/ml leupeptin, 150 mM NaCl). Protein concentrations were measured by the Bradford method (Bradford, 1976). Proteins (50 μg per lane) were size-separated in 6% (iNOS) or 8% (COX-2) and transferred a PVDF **1**membrane (Millipore, Bedford, MA, USA) by running at 60 V for 5 h. The membrane was blocked with 5% skim milk for 2 h, and then incubated for 2 h with primary antibody (iNOS, Upstate Biochemistry Inc.; COX-2, Santa Cruz Biotechnology Inc.). After washing, the membrane was incubated with the secondary antibody conjugated with horseradish peroxidase. After extensively washing the membrane, the signal was detected using the Amersham ECL system (Amersham Pharmacia Biotech, Buckinghamshire, UK). To show the amount of loading proteins, the membrane were stripped and reported with anti-tubulin (InnoGenex, San Ramon, CA, USA).

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