A New Triterpene and Anti-Hepatitis B Virus Active Compounds from *Alisma orientalis*

Zhi-Yong Jiang[1], Xue-Mei Zhang[1], Feng-Xue Zhang[2], Ni Liu[2], Fang Zhao[2], Jun Zhou[1,3], Ji-Jun Chen[1,3]

Abstract

A new triterpenoid named alisol O (**1**) was isolated from the rhizomes of *Alisma orientalis*, together with six known compounds: alisol A 24-acetate (**2**), 25-anhydroalisol A (**3**), 13β,17β-epoxyalisol A (**4**), alisol B 23-acetate (**5**), alisol F (**6**), and alisol F 24-acetate (**7**). Based on 1D and 2D-NMR data (HMQC, HMBC, COSY, ROESY), the structure of the new compound was deduced to be 11-dehydroxy-12-dehydroalisol F-24-acetate (**1**). Compounds **2–7** exhibited inhibitory activity *in vitro* on hepatitis B virus (HBV) surface antigen (HBsAg) secretion of the Hep G2.2.15 cell line with IC_{50} values of 2.3, 11.0, 15.4, 14.3, 0.6 and 7.7 μM, and on HBV e antigen (HBeAg) secretion with IC_{50} values of 498.1, 17.6, 41.0, 19.9, 8.5 and 5.1 μM, respectively.

Alisma orientalis (Sam.) Juzep. is widely cultivated in China and Japan, and its dried rhizome is a crude drug commonly used for diuretics, hypolipidemic and diabetes [1]. A series of investigations on the crude drug have revealed that the protostane-type triterpenes and sesquiterpenes are the principal constituents [2], [3], [4], [5], [6], [7], [8], [9], [10], [11], [12], [13], [14], [15]. During the course of our search for an anti-HBV active compound from plants, *A. orientalis* rhizome was investigated to afford a new triterpene, alisol O (**1**), together with six known compounds, alisol A 24-acetate (**2**), 25-anhydroalisol A (**3**), 13β,17β-epoxyalisol A (**4**), alisol B 23-acetate (**5**), alisol F (**6**), and alisol F 24-acetate (**7**). Their structures are shown in Fig. 1. Compounds **2–7** were evaluated for their anti-HBV activities *in vitro* using the HBV transfected Hep G2.2.15 cell line. The secretion of both HBV surface and e antigens by the cultured Hep G2.2.15 cells were suppressed by the application of compounds **2–7**. This paper presents the structure elucidation of the new compound and results on the anti-HBV activity of effective compounds isolated from the title plant.

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Received: November 20, 2005 · **Accepted:** May 23, 2006

Bibliography: Planta Med 2006; 72: 951–954 © Georg Thieme Verlag KG Stuttgart · New York · DOI 10.1055/s-2006-947178 · Published online July 20, 2006 · ISSN 0032-0943

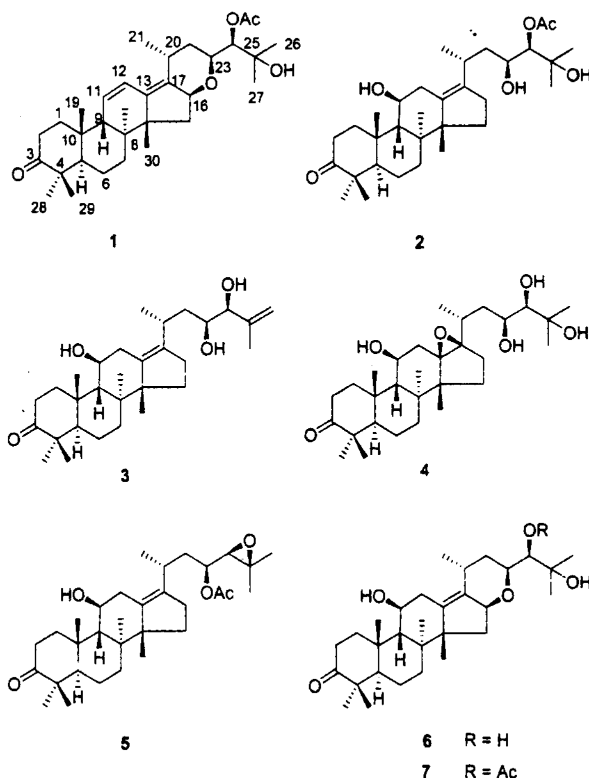

Fig. 1 Structures of compounds **1–7**.

Compound **1** was obtained as colorless prisms. The EI-MS gave a molecular ion peak at *m/z* = 512. The positive HR-ESI-MS suggested the molecular formula $C_{32}H_{49}O_5$ (513.3577 $[M + H]^+$, calcd.: 513.3580). In the IR spectrum, absorptions for hydroxy (3454 cm^{-1}), ester carbonyl (1739 cm^{-1}), ketone (1706 cm^{-1}), and olefinic functions (1630 cm^{-1}) were observed. NMR spectral analysis revealed that compound **1** should be a protostane-type triterpenoid. The NMR data of **1** were very similar to those of alisol F 24-acetate (**7**) [7], [13] except that there were two more olefinic protons at δ_H = 6.25 (1H, dd, *J* = 10.1, 3.3 Hz, H-11) and 5.68 (1H, br d, *J* = 10.1 Hz, H-12) as given in the ^{1}H-NMR spectrum (Table 1). These spectral observations suggest that compound **1** possesses one more double bond than alisol F 24-acetate, which was confirmed by the ^{13}C-NMR signals at δ_C = 120.9 and 130.2 (Table 1). The long-range correlations between H-12 and C-13, 17, H-11 and C-9 in the HMBC spectrum of **1** (Fig. 2) indicated that the additional double bond should be present at C-11(12). The HMBC spectrum also displayed a correlation between H-24 (δ_H = 4.70) and the acetyl (δ_C = 171.1) suggesting that the acetyl group was attached at C-24, together with a correlation between H-16 and C-23 demonstrating a 16,23-epoxide unit in the molecule. The configurations of H-16, H-23 and H-24 were assigned as α-orientation based on the ROESY spectrum in which correlations between H-21 and H-16, H-23, H-24 were shown. Consequently, compound **1** is deduced to be 24 (*R*)-hydroxyprotosta-11,13-diene 24-acetate 16(*S*),23(*S*)-epoxide (**1**) and named alisol O.

Table 1 ^{1}H- (500 MHz) and ^{13}C-NMR (125 MHz) data for compound **1** in $CDCl_3$

Position	^{13}C	^{1}H	Position	^{13}C	^{1}H
1	31.2 (t)	2.05 - 2.07 (m), 1.66 - 1.69 (m)	17	134.3 (s)	-
2	33.5 (t)	2.66 - 2.69 (m), 2.29 - 2.33 (m)	18	22.6 (q)	0.87 (3H, s)
3	219.5 (s)	-	19	24.7 (q)	0.91 (3H, s)
4	47.2 (s)	-	20	27.3 (d)	2.97 (1H, m)
5	46.4 (d)	2.29 - 2.33 (m)	21	17.3 (q)	1.19 (3H, d, 7.2)
6	19.3 (t)	1.48 - 1.50 (m), 1.22 - 1.30 (m)	22	35.8 (t)	1.67 (1H, ddd, 12.7,11.9, 5.3), 1.30 - 1.34
7	32.3 (t)	1.85 - 1.93 (m), 1.22 - 1,30 (m)	23	72.8 (d)	4.32 (1H, ddd, 11.9, 2.2, 2.0)
8	38.1 (s)	-	24	77.3 (d)	4.70 (1H, d, 2.2)
9	47.4 (d)	2.30 (1H, br d, 3.3)	25	72.8 (s)	-
10	35.9 (s)	-	26	26.6 (q)	1.36 (3H, s)
11	120.9 (d)	6.25 (dd, 10.1, 3.3)	27	27.9 (q)	1.12 (3H, s)
12	130.2 (d)	5.68 (br d, 10.1)	28	29.3 (q)	1.08 (3H, s)
13	139.1 (s)	-	29	19.2 (q)	1.05 (3H, s)
14	55.1 (s)	-	30	24.6 (q)	1.09 (3H, s)
15	37.0 (t)	2.17(1H,dd,14.8,7.6)	MeCO	171.1 (s)	-
16	81.0 (d)	4.57(1H, dd, 7.6, 4.8)	MeCO	20.7 (q)	2.16 (3H, s)

The anti-HBV activities of the six compounds (**2**–**7**) isolated in a large amount from *A. orentalis* in the present study were evaluated using the Hep G2.2.15 cell line stably transfected with the HBV genome. Anti-HBV activity, cytotoxicity and selectivity index (SI) are summarized in Table **2**. It was concluded that 25-anhydroalisol A (**3**), 13β,17β-epoxyalisol A (**4**), alisol B 23-acetate (**5**), and alisol F 24-acetate (**7**) showed significant anti-HBV activity at non-toxic concentrations with SI values of about 2 to 14 for HBsAg and about 1 to 2 for HBeAg at low cytotoxicity. Alisol A 24-acetate (**2**) and alisol F (**6**) showed higher SI values of 13.5 and 3.8 for HBsAg, but no activity for HBeAg at the toxic concentration, which suggested that alisol A 24-acetate (**2**) and alisol F (**6**) can specifically suppress the HBsAg secretion of the 2.2.15 cell line. Interestingly, alisol F 24-acetate (**7**) showed significant activity for both HBsAg and HBeAg with SI values of 18.5 and 28.0, suggesting that alisol F 24-acetate (**7**) should be further investigated. Unfortunately, compound **1** was obtained only in a trace amount (5 mg) and could not be evaluated for its anti-hepatitis activity. Rukachaisirikul et al. reported that some protostane-type triterpenes have an activity against HIV-1 reverse transcriptase [19], herein we have reported a series of protostane-type triterpenes possessing anti-HBV activity for the first time. These results suggest that protostane triterpenes might be of value as anti-virus agents.

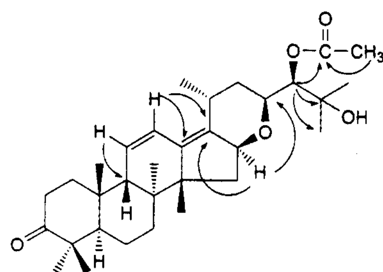

Fig. 2 Selected HMBC correlations of compound **1**.

Materials and Methods

General: Column chromatography (CC): silica gel (200–300 mesh; Qingdao Marine Chemical Inc.; Qingdao, China); Lichrospher Rp-18 gel (40–63 μ; Merck; Darmstadt, Germany). Optical rotations were carried out on a HORIBA SEPA-300 High Sensitive Polarimeter. IR spectra were measured on a Bio-Rad FTS-135 spectrometer with KBr pellets, ν in cm^{-1}. MS data were obtained on a VG Auto Spec-3000 instrument. NMR spectra were recorded on Bruker AM 400 (^{1}H/^{13}C, 400 MHz/100 MHz) or DRX-500 (^{1}H/^{13}C, 500 MHz/125 MHz) spectrometers and chemical shifts were given in δ with TMS as internal reference.

Plant material: The dried rhizomes of *A. orientalis* were collected in Sichuan province in October 2002 and identified by Prof. Jun Zhou, Kunming Institute of Botany, Chinese Academy of Sciences, where a voucher specimen (K 2002 - 10 - 008) was deposited.

Table **2** Anti-HBV activity, cytotoxicity and selectivity index of compounds **2**–**7**

Compounds	CC_{50} (μM)	HBsAg IC_{50} (μM)	HBsAg SI	HBeAg IC_{50} (μM)	HBeAg SI
2	31.1	2.3	13.5	498.1	0.06
3	23.0	11.0	2.1	17.6	1.3
4	59.8	15.4	3.9	41.0	1.4
5	26.3	14.3	1.8	19.9	1.3
6	2.3	0.6	3.8	8.5	0.27
7	142.7	7.7	18.5	5.1	28.0
ADFV	> 1.0 (mM)	0.06 (mM)	> 16.7	0.1 (mM)	> 10.0

ADFV: adefovir dipivoxil, an antiviral agent used as positive control. HBsAg: HBV surface antigen. HBeAg: HBV e antigen.

Extraction and isolation: The dried rhizomes (9.0 kg) were powdered and extracted with 90% EtOH (10 L×3) under reflux. The extract was concentrated under vacuum to give a residue which was partitioned between water, $CHCl_3$ and *n*-BuOH, respectively, to provide a $CHCl_3$ fraction (380 g) and an *n*-BuOH fraction (20 g). The $CHCl_3$ fraction was fractionated by silica gel CC (2.0 kg, 200–300 mesh) with gradient elution with $CHCl_3$/MeOH ($CHCl_3$/MeOH 100:0, 98:2, 95:5, 90:10, 85:15, 80:20, each 4 L). According to the chemical distinctions revealed by TLC, five crude fractions (A - E) were obtained. Fr.B (23g) was subjected to a silica gel CC (300 g) and eluted with $CHCl_3$/MeOH (98:2) to provide Frs.B1 – 4. Fr.B3 (3.8 g) was chromatographed over a silica gel column (100g, $CHCl_3$/MeOH 98:2) to afford three fractions (Fr.B3.1 – 3). Fr.B3.2 (0.5 g) was chromatographed over a silica gel column (50 g) eluted with $CHCl_3/Me_2CO$ (90:10) to provide two sub-fractions (Fr.B3.2.1 and Fr.B3.2.2),.Fr.B3.2.2 (0.2 g) was further purified by RP-18 CC (50 g, MeOH/H_2O, 80:20) to yield compound **1** (R_f = 0.6, RP-18 TLC, MeOH/H_2O, 90:10, 5 mg).

Alisol O (**1**): Colorless prisms (MeOH); m.p. 148–151.5 °C; $[\alpha]_D^{17}$: +20.6° (*c* 0.6, $CHCl_3$); IR (KBr): ν_{max} = 3454, 1739, 1706, 1630, 1242, 1053 cm^{-1}; EI-MS (70 eV): *m/z* = 512 (37), 494 (10), 452 (2), 381 (43), 203 (95), 109 (100); HR-ESI-MS: *m/z* = 513.3577 $[M + H]^+$ (calcd. for $C_{32}H_{48}O_5$: 513.3580); ^1H- and ^{13}C-NMR ($CDCl_3$) data, see Table **1**.

Anti-HBV assay: The anti-HBV activity assay was performed according to the previous reports [16], [17]. Briefly, the compounds used in the present study were evaluated in the 2.2.15 cell line which was stably transfected (Lipofectamine 2000 reagent; Invitrogen; Carlsbad, CA, USA) with the HBV genome. The toxicity of the compounds was assayed by a modified MTT (GIBCO Invitrogen; Carlsbad, CA, USA) method [18]. DMSO (GIBCO) alone was added to each culture as a solvent control. All the evaluated compounds were dissolved in DMSO. The concentration of DMSO in the media was maintained at less than 2.5 μL/mL to ensure that it did not affect the growth of 2.2.15 cells. The sub-toxic concentration of the identified compounds was measured with a serial dilution in 96-well microplates in which cells were seeded at a density of 3×10^4/mL and cultured at 37 °C, 5% CO_2 for 12 days. After incubation, the cells and supernatants were collected. The levels of HBsAg and HBeAg in the supernatants were assayed with an ELISA (Sino-American Biotech.; Luoyang, China) method. The absorbance (*A*) of each well was measured at 490 nm using a microplate reader (ELX800; Bio-Tek Instruments Inc.; Winooski, VT, USA). The 50% inhibitory concentration (IC_{50}) and 50% cytotoxic concentration (CC_{50}) were determined as follows:

$$\eta_{destroy} = (A_{cellcontrol} - A_{experimental})/(A_{cellcontrol} - A_{tblank}) \times 100$$

$$\eta_{inhibitory} = (A_{cellcontrol} - A_{experimental})/(A_{cellcontrol} - A_{tblank}) \times 100$$

$$SI = \eta_{destroy}/\eta_{inhibitory}$$

An antiviral agent, adefovir dipivoxil (ADFV; The Academy of Military Medical Sciences; Beijing, China), was used as a positive control.

Cell line and cell culture: The Hep G2.2.15 cell line is widely used for the screening of anti-HBV drugs. In this study, 2.2.15 cells established from a hepatoma cell line Hep G2 (ATCC; Manassas, VA, USA) were cultured in RPMI-1640 (GIBCO) medium supplemented with 10% fetal calf serum (GIBCO), 100 μg/mL G148 (GIBCO), 100 IU/ mL penicillin (GIBCO), 100 IU/ mL streptomycin (GIBCO). All cultures were maintained at 37 °C in a moist atmosphere containing 5% CO_2.

Cytotoxicity assay: The toxicity of the compounds was assayed by a modified MTT method [18]. In brief, the test samples were prepared at different concentrations. After Hep G2.2.15 cells had been seeded in a 96-well microplate for 4 hours, the samples (20 μL) were placed in each well and incubated for 3 days in 37 °C; then, 0.1 mL MTT [3-(4,5-dimethylthiazole-2-yl)-2,5-diphenyltetrazolium bromide, 400 μg/mL] was added for 4 hours. After removal of the MTT medium, DMSO (100 μL/well) was added onto the microplate for 10 min. The formazan crystals were dissolved, and the absorbance was measured on a microplate reader at 490 nm.

Acknowledgements

The authors are grateful to the members of the analytical group of the State Key Laboratory of Phytochemistry and Plant Resources in West China, Kunming Institute of Botany, Chinese Academy of Sciences, for measurements of all spectra.

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Stability of Andrographolide in Powdered Andrographis Herb under Accelerated Conditions

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Abstract

The stability of andrographolide in powdered Andrographis Herb – the aerial part of *Andrographis paniculata* (Burm. f.) Nees (Acanthaceae) – was determined using a heat-accelerated experiment to reveal a second-order kinetics of degradation. The fast decomposition was observed regardless of the method of analysis. The rate constant of the decomposition of andrographolide at 25 °C ($k_{25\,°C}$), predicted from the Arrhenius plot, was 6.58×10^{-6} d^{-1}.

Supporting information available online at http://www.thieme-connect.de/ejournals/toc/plantamedica

Andrographis Herb (APH) or Andrographitis Herba, as officially named in the Thai Herbal Pharmacopoeia (THP), is the dried aerial part of *Andrographis paniculata* (Burm. f.) Nees (Acanthaceae) [1]. The plant is widely known for its wide range of activities (for example, see [2], [3], [4], [5]) and is used in several Asian countries including China, India, and Thailand. In THP [1], APH is categorized as an anti-inflammatory agent for laryngitis, as well as an antidiarrheal and antipyretic agent. The herb is also used for the treatment of liver and cardiovascular diseases in Ayurvedic medicines [6], [7]. The active constituents in *A. paniculata* responsible for the activities are labdane-type diterpene lactones, among which andrographolide (**1**) is the major component (Fig. **1**) [8].

Despite its high potential, *A. paniculata* is one of a few herbal medicines associated with a short shelf life. The shelf life of 12 months was recommended by THP [1], as estimated according to the decrease in the total lactone content by 26% upon 1-year storage of the dried, powdered herb in dry, ambient conditions [9]. The second-order degradation of amorphous andrographolide under a heat-accelerating condition corresponded well with such a recommendation [10]. On the other hand, when in aqueous solution, **1** decomposed through a first-order kinetics

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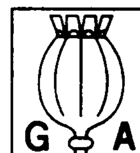
Received: January 17, 2006 · **Accepted:** May 31, 2006

Bibliography: Planta Med 2006; 72: 954–956 © Georg Thieme Verlag KG Stuttgart · New York · DOI 10.1055/s-2006-946696 · Published online August 10, 2006 · ISSN 0032-0943



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