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Anti-HIV-1 protease triterpenoids from *Stauntonia obovatifoliola* Hayata subsp. *intermedia*

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HIV-1 protease inhibitors

ABSTRACT

Three triterpenoids, 16β-hydroxy-2,3-*seco*-lup-20(29)-ene-2,3-dioic acid (**1**), 3β,21β,24-trihydroxy-30-noroleana-12,20(29)-dien-28-oic acid (**2**) and 16β-hydroxylupane-1,20(29)-dien-3-one (**3**), along with eleven known triterpenes were isolated from stems of *Stauntonia obovatifoliola* Hayata subsp. *intermedia* (Y.C. Wu) T. Chen. Their structures were determined by analysis of HR-EI/FAB-MS and 1D and 2D NMR spectroscopic data and comparison with those in the literature. Ten of the compounds showed inhibitory activity against HIV-1 protease.

1. Introduction

The literature contains a fairly large number of articles dealing with the constituents, mainly triterpenes and lignans, of *Stauntonia chinensis* and *Stauntonia hexaphylla* (Lardizabalaceae) (Ikuta, 1989; Wang et al., 1989, 1993; Gao et al., 2007) some of which have been reported to have antitumor and cytotoxic properties (Liu et al., 2004; Nakanishi et al., 2005). However, there is no report on the chemical constituents of *S. obovatifolia*. Both it and its subspecies are widely used for their analgesic and sedative effects (Jiangsu New Medical College, 1977). In the course of our search for anti-HIV agents among traditional Chinese medicines, we investigated the stems of *Stauntonia obovatifoliola* subsp. *intermedia* for isolation of inhibitory substances against HIV-1 protease, and isolated fourteen compounds (**1**–**14**), including three new triterpenoids named 16β-hydroxy-2,3-*seco*-lup-20(29)-ene-2,3-dioic acid (**1**), 3β,21β,24-trihydroxy-30-noroleana-12,20(29)-dien-28-oic acid (**2**) and 16β-hydroxylupane-1,20(29)-dien-3-one (**3**). Their structures were determined by spectroscopic means including use of 2D NMR spectroscopy. In this paper, we report the isolation, structural determination and bioactivity of these compounds.

2. Results and discussion

The dried stems of *S. obovatifoliola* Hayata subsp. *intermedia* were extracted with EtOH–H_2O (3:1, v/v) at room temperature. The combined extracts were concentrated to dryness to afford a crude extract, which was suspended in water and extracted with EtOAc and BuOH. The EtOAc extract was subjected to column chromatography on silica gel, octadecylsilanized (ODS) silica gel and HPLC, to yield compounds **1**–**14** (Fig. 1). Compounds **4**–**14** were identified as 3β-hydroxy-30-noroleana-12,20(29)-dien-28-oic acid (**4**) (Ikuta and Itokawa, 1986), 3α,24-dihydroxy-30-noroleana-12,20(29)-dien-28-oic acid (**5**) (Ikuta and Itokawa, 1988), lupeol (**6**) (Wenkert et al., 1978), lupenone (**7**) (Dantanarayana et al., 1982), resinone (**8**) (Pech et al., 2002), lup-20(29)-ene-3β,16β-diol (**9**) (Wenkert et al., 1978), lup-20(29)-ene-3β,28-diol (betulin) (**10**) (Sholichin et al., 1980), hederagenin 3-*O*-α-L-arabinopyranoside (**11**) (Kim et al., 1990), 3-*O*-acetyloleanolic acid (**12**) (Ikuta, 1989), mesenbryanthemoidgenic acid (**13**) (Ikuta and Itokawa, 1986), 3β,23-dihydroxy-olean-12-en-28-oic acid (**14**) (Tori et al., 1974). The structures of the new compounds, **1**, **2** and **3**, were determined as follows.

Compound **1** was isolated as a colorless amorphous solid. Its negative electrospray ionization mass spectrum (ESI-MS) exhibited a quasi-molecular ion peak at *m*/*z* 487.2 ($[M-H]^-$), indicating a molecular weight of 488.2. The molecular formula was established as $C_{30}H_{48}O_5$ by the negative-ion mode HR-FAB-MS showing a

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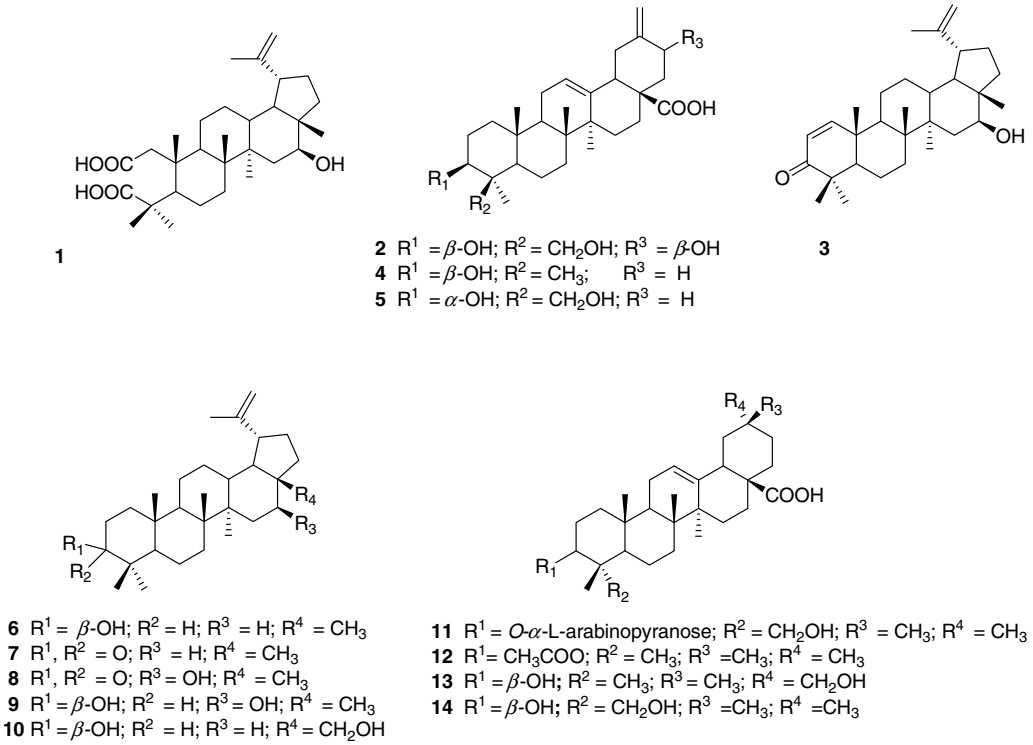

Fig. 1. Structures of isolated compounds.

pseudo-molecular ion peak at *m*/*z* 487.34246, a compound with 7 degrees of unsaturation. The ^{1}H NMR spectrum of **1** (Table 1) displayed signals for typical triterpenoid methyl groups at δ 0.80 (3H, H_3-28), 0.96 (3H, H_3-25) 1.04 (3H, H_3-27), 1.09 (3H, H_3-26), 1.24 (3H, H_3-23), 1.26 (3H, H_3-24) and 1.68 (3H, H_3-30) [δ_C 12.2, 20.5, 16.7, 16.5, 27.3, 24.9 and 19.5, respectively, according to the HMQC experiment]. The presence of a broad vinyl methyl proton signal at δ 1.68 and two vinyl proton signals at δ 4.71 (*d*, *J* = 2.0 Hz) and 4.59 (d, *J* = 2.0 Hz) with ^{13}C signals at δ 19.5, 151.3 and 110.4 was characteristic of an isopropenyl group of lupene triterpenes. The ^{1}H NMR spectrum also suggested a double doublet signal at δ 3.54 (*J* = 11.5, 4.5 Hz, H-16) due to a hydroxymethylene group (δ_C 77.5). The ^{13}C NMR spectrum (Table 1) and DEPT experiments indicated the presence of 30 carbon atoms due to seven methyls, nine methylenes, six methines, and eight non-protonated carbons. The signals included two olefinic carbons [δ 110.4 (C-29), 151.3 (C-20)] and two carbonyl carbons [δ 175.5 (C-2), 183.6 (C-3)]. The ^{1}H-detected heteronuclear multiple bond connectivity (HMBC) correlations of H_2-1 to one carboxyl carbon indicated that the carboxyl group (δ 175.5) was adjacent to C-1. The long-range correlation of two methyl groups (H_3-23 and H_3-24) to another carboxyl carbon (δ_C 183.6) suggested that the second carboxyl group was adjacent to C-4. Moreover, the HMBC correlations between a carbon signal at δ_C 77.5 (C-16) and a methyl proton signal at δ 0.80 (H_3-28), as well as between a proton signal at δ 3.54 (H-16) and a carbon signal at δ_C 45.5 (C-14), indicated that a hydroxyl group was attached at C-16. The orientation of the hydroxyl group was determined by the NOESY experiment in which the proton signal (H-16) was found to have correlation with a methyl signal (H_3-27, α-orientation). The presence of two carboxyl groups at C-2 and C-3 led us to conclude that the structure was 16β-hydroxy-2,3-*seco*-lup-20(29)-ene-2,3-dioic acid (**1**). Although many *seco*-triterpenoids, especially 3,4-*seco*-compound, have been reported as natural products (Baas, 1985), compound **1** is the first 2,3-*seco*-triterpenoid from the plant family *Lardizabalaceae*.

Compound **2** was isolated as an amorphous powder. Its negative ESI-MS spectrum exhibited a quasi-molecular ion peak at *m*/*z* 471 ($[M-H]^-$). The molecular formula $C_{29}H_{44}O_5$ was assigned by HR-EI-MS (positive ion mode, *m*/*z* 472.31874). The compound showed 8 degrees of unsaturation. Analysis of connectivities in the ^{1}H–^{1}H COSY and HMBC spectra confirmed that two of the 8 unsaturations were due to double bonds; one was due to a carboxyl group and the remaining five were due to five rings. The ^{1}H NMR spectrum of **2** (Table 1) showed signals for four tertiary methyl groups [δ 0.81 (3H, H_3-25), 0.93 (3H, H_3-26), 1.25 (3H, H_3-27), 1.56 (3H, H_3-23)], two exo-methylene protons at [δ 5.08 (H_a-29), 5.61 (H_b-29)], an olefinic proton at δ 5.53 (1H, H-12), one proton at δ 3.64 assigned to H-3, two protons [δ 3.63 (H_a-24), 4.47 (H_b-24)] ascribable to hydroxymethyl protons, respectively, and an H-21 proton signal appeared at δ 4.76. The ^{13}C NMR spectrum of **2** showed 29 carbon signals which suggested a noroleanane skeleton. In the HMBC spectrum of **2**, 20(29)-exo-methylene proton signals at δ 5.08 (H_a-29) and 5.61 (H_b-29) correlated with one methylene and one hydroxymethylene carbon resonances at δ 42.2 (C-19) and 68.0 (C-21), respectively, which further suggested the skeleton to be a noroleanane type. Two carbon signals at δ 152.9 (C-20) and 104.2 (C-29) were assigned to the exo-methylene by analysis of the HMBC spectrum (key correlations are shown in Fig. 2). Two olefinic carbons at δ 123.1 (C-12) and δ 143.5 (C-13) indicated a typical Δ^{12} oleanane-type pentacyclic triterpene. The signals at δ 80.1 and 64.5 were further assigned to C-3 and to C-24 (hydroxymethyl carbon), respectively. The correlations of δ_H 1.56 (H_3-23) and δ_H 3.63, 4.47 (H_2-24) with one hydroxyl bearing carbon δ_C 80.1 (C-3) suggested that the hydroxyl group was attached to C-3. The long-range correlations of signals at δ 2.46 (H_2-19), δ 2.22 (H_2-22) and δ 5.08 (H_2-29) with a carbon signal at δ 68.0 (C-21), indicated the second hydroxyl group was attached to C-21, and of an H_2-24 signal with resonances at δ_C 80.1 (C-3), δ_C 56.3 (C-5) and δ_C 23.6 (C-23), established that the third hydroxyl group was attached to C-24. The orientation of three hydroxyl groups were con-

Table 1
NMR spectroscopic data for compound **1** (Methanol-d_4), compound **2** (pyridine-d_5) and compound **3** ($CDCl_3$)

No.	**1**		**2**		**3**	
	δ_H	δ_C	δ_H	δ_C	δ_H	δ_C
1	a, 2.43 (*d*, 17.5)[a]	43.1	0.93 (*m*)[a]	38.6	7.11 (*d*, 10.0)	159.9
	b, 2.57 (*d*, 17.5)		1.44 (*m*)[b]			
2		175.5	1.16 (*m*)[c]	28.3	5.81 (*d*, 10.0)	125.1
			1.98 (*m*)			
3		183.6	3.64 (*dd*, 6.0, 12.0)[d]	80.1		205.8
4		47.7		43.1		44.7
5	2.50 (*m*)[b]	49.0	0.94 (*m*)[a]	56.3	1.54 (*m*)[a]	53.4
6	1.94 (*m*)	30.9	a, 1.32 (*m*)[e]	19.0	a, 1.22 (*m*)	19.0
	1.96 (*m*)		b, 1.65 (*m*)[f]		b, 1.85 (*m*)	
7	a, 1.38 (*m*)	34.6	a, 1.26 (*m*)	33.5	1.48 (*m*)	33.7
	b, 1.53 (*m*)		b, 1.44 (*m*)[b]		1.54 (*m*)[a]	
8		41.9		39.7		44.3
9	2.44 (*m*)[a]	43.2	1.65 (*m*)[f]	47.7	1.58 (*m*)[b]	44.0
10		42.0		37.0		39.5
11	a, 1.46 (*m*)	22.4	a,1.30 (*m*)[e]	25.1	a, 1.38 (*m*)[c]	21.1
	b, 1.63 (*m*)[c]		b, 2.23 (*m*)[g]		b, 1.64 (*m*)[d]	
12	a, 1.19 (*m*)	23.0	5.53 (t, 3.5)	123.1	a, 1.09 (*m*)	24.7
	b, 1.23 (*m*)				b, 1.77 (*m*)	
13	1.64 (*m*)[c]	38.9		143.5	1.58 (*m*)[b]	36.6
14		45.5		42.1		44.3
15	a, 1.30 (*m*)	37.7	a, 1.16 (*m*)[c]	28.4	a, 1.28 (*m*)	37.7
	b, 1.57 (*m*)		b, 1.96 (*m*)		b, 1.66 (*m*)[d]	
16	3.54 (*dd*, 11.5, 4.5)	77.5	a, 1.80 (*m*)	24.0	3.65 (*dd*, 4.5, 11.5)	77.3
			b, 1.92 (*m*)			
17		45.5		48.3		48.7
18	1.43 (*m*)	49.2	3.35 (*dd*, 4.0, 13.5)	48.8	1.41 (*m*)	47.5
19	2.51 (*m*)[b]	48.7	a, 2.46 (*dd*, 4.0 13.5)	42.2	2.52 (dt, 6.0, 11.0)	47.5
			b, 2.82 (*dd*, 4.0, 13.5)			
20		151.3		152.9		149.7
21	a, 1.04 (*m*)[d]	26.3	4.76 (*dd*, 4.0, 10.5)	68.0	a, 1.39 (*m*)[c]	29.8
	b, 1.75 (*m*)				b, 1.99 (*m*)	
22	a, 1.28 (*m*)	39.0	a, 2.22 (*m*)[g]	48.0	a, 1.31 (*m*)	37.4
	b, 1.68 (*m*)[e]		b, 2.75 (*m*)		b, 1.72 (*m*)[e]	
23	1.24 (*s*)	27.3	1.56 (*s*)	23.6	1.10 (*s*)	27.8
24	1.26 (*s*)	24.9	a, 3.63 (*d*, 10.0)[d]	64.5	1.05 (*s*)	21.4
			b, 4.47 (*d*, 10.0)			
25	0.96 (*s*)	20.5	0.81 (*s*)	15.9	1.03 (*s*)	19.2
26	1.09 (*s*)	16.5	0.93 (*s*)	17.2	1.14 (*s*)	16.4
27	1.04 (*s*)[d]	16.7	1.25 (*s*)	25.9	0.95 (*s*)	16.0
28	0.80 (*s*)	12.2		178.7	0.83 (*s*)	11.7
29	a, 4.59 (*d*, 2.0)	110.4	5.08 (*m*)	104.2	4.62 (*br s*)	110.0
	b, 4.71 (*d*, 2.0)		5.61 (*m*)		4.73 (*br d*, 1.5)	
30	1.68 (*br s*)[e]	19.5			1.70 (*s*)[e]	19.3

[a–g] Signals bearing the same superscript overlapped.

firmed by NOESY, in which the following correlations were found, δ 2.82 (H_α-19) to δ 1.25 (H_3-27) and δ 4.76 (H-21) to H_α-19 (δ 2.82), indicated that H-21 is an α-proton and thus OH-21 is β-orientated; a correlation between δ 3.35 (H-18) and δ 2.46 (H_β-19) demonstrated that H-18 is an β-orientation; a correlation of δ 3.63, 4.47 (H_2-24) with δ 0.81 (H_3-25) indicated that a hydroxymethyl group was at position C-24 and a correlation of δ 3.64 (H_1-3) with δ 0.94 (H-5), showed that OH-3 is β-orientated.

Based on the above evidence, the structure of **2** was determined as 3β,21β,24-trihydroxy-30-noroleana-12,20(29)-dien-28-oic acid.

Compound **3** was isolated as white powder. Its molecular formula was established as $C_{30}H_{46}O_2$ by the HR-EI-MS showing a molecular ion peak at *m*/*z* 438.35079 $[M]^+$. The ^1H NMR spectrum of **3** (Table 1) showed characteristic signals of seven methyl groups at δ 0.83 (3H, H_3-28), 0.95 (3H, H_3-27) 1.03 (3H, H_3-25), 1.10 (3H, H_3-23), 1.14 (3H, H_3-26), 1.05 (3H, H_3-24) and 1.70 (3H, H_3-30); proton resonances for one isopropenyl group at δ 1.70, δ 4.62 and δ 4.73; two olefinic proton signals at δ 5.81 (*d*, *J* = 10.0 Hz) and δ 7.11 (*d*, *J* = 10.0 Hz), implying an α,β-unsaturated ketone; one double doublet resonance at δ 3.65 (*J* = 4.5, 11.5 Hz) due to a hydroxymethine group (δ_C 77.3). The ^{13}C NMR spectrum (Table 1) indicated the presence of 30 carbon atoms, including resonances at δ 19.3 (C-30), 110.0 (C-29), 149.7 (C-20) as well as at δ 125.1 (C-2), 159.9 (C-1) and 205.8 (C-3) for the isopropenyl and α,β-unsaturated carbonyl groups, respectively. The HMBC correlations observed between a proton signal at δ 7.11 (H-1) and carbon resonances at δ 205.8 (C-3), 53.4 (C-5) and 39.5 (C-10), respectively, along with the HMBC correlations from δ 5.81 (H-2) to δ 44.7 (C-4) and δ 39.5 (C-10) indicated that the double bond was at C-1 and C-2 positions. This was further supported by HMBC correlations of δ 1.03 (H_3-25) with δ 159.9 (C-1), 53.4 (C-5) and 39.5 (C-10). From the long-range correlations between methyl group signals at δ 1.10 (H_3-23) and δ 1.05 (H_3-24) and a carbonyl carbon signal at δ_C 205.8 (C-3), an oxo group was established to be located at C-3. The HMBC correlations between a methyl at δ 0.83 (H-28) and carbons at δ_C 77.3 (C-16), δ_C 37.4 (C-22) as well as between δ 3.65 (H-16) and δ_C 37.7 (C-15), δ_C 47.5 (C-18) and δ_C 48.7 (C-17), indicated that a hydroxy group must be at C-16. The β-orientation of the hydroxyl group was indicated by a correlation of the H-16 proton with H_3-27 (α) in the NOESY spectrum (Fig. 2). Based on the above evidence, compound **3** was determined as 16β-hydroxylupane-1,20(29)-dien-3-one.

In our continuing search for potential anti-HIV natural products (Ma et al., 2003), we carried out anti-HIV-1 protease activity tests

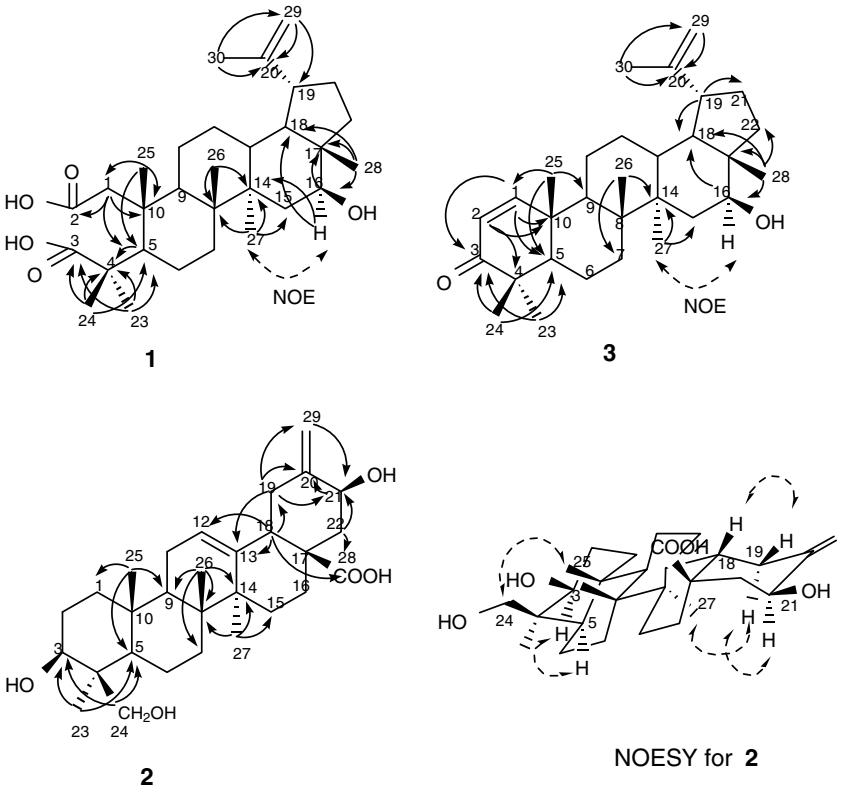

Fig. 2. Selected HMBC correlations for **1**, **2** and **3** as well as NOESY for **2**.

for these 14 compounds. Ten of them showed inhibitory effects greater than 50% at 100 μg/ml (Table 2). Notably, compound **1** showed an inhibitory effect of 100 ± 0.0% at this concentration. IC_{50} of compound **1** was determined as 8.7 μg/ml in this assay. A known HIV-1 protease inhibitor, oleanolic acid had an IC_{50} of 24.8 μg/ml in the same experiment.

3. Concluding remarks

We found in this experiment that 2,3-*seco*-lupane, 30-norolean-ane, lupane and oleanane type triterpenes accumulated in the stems of *S. obovatifoliola* subsp. *intermedia* with compounds **6**–**8** being the major compounds. Interestingly, compound **1** possessing a novel 2,3-*seco*-2,3-dioc acid moiety in ring A showed potent inhibitory activity against HIV-1 protease, indicating that the 2,3-*seco*-2,3-dioc acid moiety in ring A might be an important pharmacophore to be considered for designing and synthesizing HIV-protease inhibitors. Since some triterpene derivatives with a 3-*O*-acidic acyl group showed strong anti-HIV activity (Kashiwada et al., 1996), and 3-*O*-(3′,3′-dimethylsuccinyl) betulinic acid (PA-457, bevirimat) was reported to enter phase 2 clinic trial as a new AIDS drug candidate (Beatty et al., 2005; Panacos, 2007), it is of interest to examine anti-HIV activity of compound **1** and other *seco*-triterpenes with acidic groups at positions 2 and 3 of A ring. The synthesis of other *seco*-triterpenes and their HIV-protease inhibitory activity are being carried out in our laboratory and will be reported later.

Table 2
Inhibitory activity of isolated compounds against HIV-1 protease

Compound	%Inhibition at 100 μg/ml	IC_{50} (μg/ml)
2,3-Seco-lupane		
1	100.0 ± 0.0%	8.7
30-Nor-oleanane type		
2	34.3 ± 0.0%	> 100.0
4	78.2 ± 1.2%	35.0
5	72.5 ± 1.5%	40.8
Lupane type		
3	82.5 ± 2.0%	25.0
6	48.5 ± 1.8%	>100.0
7	2.4 ± 0.6%	>100.0
8	85.4 ± 2.3%	29.4
9	63.8 ± 2.7%	33.0
10	64.7 ± 6.7%	53.0
Oleanane type		
11	−2.6 ± 3.2%	>100.0
12	87.4 ± 1.1%	38.0
13	88.4 ± 0.7%	28.0
14	82.5 ± 3.2%	36.0
Oleanolic acid[a]	96.0 ± 1.0%	24.8

Data represent the means ± standard deviation values from at least three separate experiments.
[a] Oleanolic acid was used as a positive control.

4. Experimental

4.1. General experimental procedures

Optical rotations were measured with a DIP-360 automatic polarimeter. UV spectra were measured with a SHIMADZU UV-2200 UV–VIS recording spectrophotometer. IR spectra were measured with a JASCO FT/IR-230 infrared spectrometer. NMR spectra were recorded on either JEOL JHA-LAA 400 WB-FT (^{1}H, 400 MHz; ^{13}C, 100 MHz) or Varian UNITY Plus 500 (^{1}H, 500 MHz; ^{13}C, 125 MHz) spectrometers. Conventional pulse sequences were used for COSY, HMBC and HMQC. All chemical shifts (δ) are given in ppm units with reference to tetramethylsilane (TMS) as an internal standard and the coupling constants (J) are in Hz. HR-EI-MS and EI-MS were taken on a JMX-AX 505 HAD gc/ms system and a JMS DX-300 system at ionization voltage of 70 eV. TLC was carried out on precoated silica gel 60 F_{254} (Merck). The following chromatography suppliers were used for isolation: silica gel BW-820MH

(Fuji, Silysia), ODS DM 1020T (Fuji Silysia), and Sephadex LH-20 (GE Healthcare Bio-sciences AB, Sweden). Prep HPLC was performed on a Tosoh CCPM-CCPM-II system (Tosoh Co.) equipped with a UV 8020 detector and a TSK gel ODS-80Ts column (21.5 × 300 mm, Tosoh Co.).

4.2. Plant material

The stems of *S. obovatifoliola* Hayata subsp. *intermedia* were collected in March, 2003 at Gaotun Town, Liping County, Guizhou Province of China and identified by Professor De-yuan Chen, botanist. The specimen of the plant (voucher no. 0303/YMG) is deposited in Guiyang College of Traditional Chinese Medicine.

4.3. Extraction and isolation

Dried and finely powdered stems of the plant (10 kg) were macerated with EtOH–H_2O (3:1, v/v) at room temperature for 48 h and the process was repeated twice. The EtOH extract was concentrated under reduced pressure and suspended in H_2O, extracted successively with EtOAc (5 l) and BuOH (5 l). The EtOAc extract (200 g) was then subjected to silica gel CC (80 cm × 6.5 cm) eluted with $CHCl_3$–MeOH (100:1, v/v → MeOH) to yield fractions A–O. Fraction A was subjected to silica gel CC (65 cm × 5 cm) eluted with $CHCl_3$–MeOH (100 → 80:1) to obtain **6** (948 mg) and **7** (1.25 g). Fractions B–C were applied to a silica gel column (80 cm × 7 cm) and eluted with hexane–acetone (10:1 → 6:1) to furnish compounds **3** (12 mg), **8** (1.43 g), **9** (171 mg), **10** (148 mg), and **12** (125 mg). Fraction D was separated using ODS open CC (20 cm × 4.5 cm) (50–80% MeOH) and prep HPLC to obtain compounds **1** (10 mg), **13** (30 mg) and **14** (15 mg). Fractions E–G were subjected to silica gel CC eluted with $CHCl_3$–MeOH–H_2O (9:1:0.1–8:2:0.2) and further purified using ODS CC (20 cm × 2.5 cm) with 50–80% MeOH and then HPLC [MeOH–0.1% TFA/H_2O, 5 ml/min, monitored at 208 nm] to obtain compounds **2** (2 mg), **4** (1 mg) **5** (4 mg) and **11** (25 mg), respectively.

4.4. Anti-HIV-protease assay

HIV-protease assay kits (Bachem Feinchemikalien AG, Bubendorf, Switzerland) were used. Twenty five microliter of HIV-protease assay buffer (50 mM NaOAc, pH 4.9) containing 2.5 μg of the substrate [His-Lys-Ala-Arg-Val-Leu-(*p*NO_2-Phe)-Glu-Ala-Nle-Ser-NH_2] was mixed with 2 μl of a compound solution (using DMSO as a solvent), then 8 μl of recHIV-protease (0.01 mg/ml) was added. The reaction mixture was incubated for 30 min at 37 °C and then terminated by addition of 3.0 μl of 10% trifluoroacetic acid (TFA). The hydrolysate (*p*NO_2-Phe-Glu-Ala-Nle-Ser-NH_2) and the remaining substrate were quantitatively analyzed by reversed-phase HPLC. HPLC conditions: column, COSMOSIL Packed 5C_{18}-MS-II, 4.6 × 150 mm NACALAI TESQUE.INC. Kyoto, Japan; solvent, gradient CH_3CN: H_2O (containing 0.1% TFA) (20–40%); flow rate, 1.0 ml/min; detector, UV 208 nm. The substrate and the hydrolysate were eluted at 8.1 and 3.5 min, respectively. The HIV-protease inhibitory activity of a compound was calculated as follows:

$$\%\ \text{inhibition} = (A_{\text{control}} - A_{\text{sample}}) \times 100/A_{\text{control}}$$

where *A* is relative peak area of the hydrolysate.

Oleanolic acid was used as a positive control, its IC_{50} being 24.8 μg/ml.

4.5. 16β-Hydroxy-2,3-seco-lup-20(29)-ene-2,3-dioic acid (**1**)

Colorless amorphous solid; $[\alpha]_D^{26} = +18.2$ ($c = 0.13$, methanol); UV λ_{max}^{MeOH} nm ($\log\varepsilon$): 202 (4.9); IR υ_{max}^{KBr} cm^{-1}: 3420, 2950, 2650, 1710, 1630; MS (ESI, negative ion mode), *m*/*z* 487.2 $[M-H]^-$, HR-FAB-MS $[M-H]^-$ *m*/*z* 487.34246 (calcd. for $C_{30}H_{47}O_5$, requires 487.34235); For ^1H and ^{13}C NMR spectroscopic data, see Table 1.

4.6. 3β,21β,24-Trihydroxy-30-noroleana-12,20(29)-dien-28-oic acid (**2**)

Colorless amorphous solid; $[\alpha]_D^{26} = +144$ ($c = 0.10$, methanol); UV λ_{max}^{MeOH} nm ($\log\varepsilon$): 213 (3.5); IR υ_{max}^{KBr} cm^{-1}: 3430, 2930, 2700, 1690, 1630; MS (ESI, negative ion mode), *m*/*z* 471.4 $[M-H]^-$, HR-EI-MS *m*/*z* 472.31874 (calcd. for $C_{29}H_{44}O_5$, requires 472.31887); For ^1H and ^{13}C NMR spectroscopic data, see Table 1.

4.7. 16β-hydroxylupane-1,20(29)-dien-3-one (**3**)

Colorless white solid; $[\alpha]_D^{27} = +98.9$ ($c = 0.065$, methanol); UV λ_{max}^{MeOH} nm ($\log\varepsilon$): 205 (4.8), 228 (4.8); IR υ_{max}^{KBr} cm^{-1}: 3500, 2930, 1690, 1620; HR-EI-MS (EI, 70 eV positive ion mode), *m*/*z* 438.35079 (calcd. for $C_{30}H_{46}O_2$, requires 438.34978); For ^1H and ^{13}C NMR spectroscopic data, see Table 1.

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