ent-Labdane Diterpenoid Lactone Stereoisomers from *Andrographis paniculata*

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Received August 23, 2007

Two pairs of *ent*-labdane diterpenoid lactone stereoisomers (**1**−**4**) including three new compounds (**1**−**3**) were isolated from the 85% EtOH extract of the aerial parts of *Andrographis paniculata*. The structures of these compounds were identified as 7*R*-hydroxy-14-deoxyandrographolide (**1**), 7*S*-hydroxy-14-deoxyandrographolide (**2**), 12*S*,13*S*-hydroxyandrographolide (**3**), and 12*R*,13*R*-hydroxyandrographolide (**4**) by spectroscopic data analyses and calculated ^{13}C NMR data at the B3LYP/6-311++G(2d,p)//B3LYP/6-31G* level using the GIAO method. The 12*S*-configuration of **4** was revised to 12*R* based on the spectroscopic data. The antiproliferative activities of the two pairs of stereoisomers and 14 other *ent*-labdane diterpenoid derivatives were determined in human leukemia HL-60 cells. Andrographolide (**7**) and isoandrographolide (**12**) exhibited higher antiproliferative activities than other *ent*-labdane diterpenoids with GI_{50}'s of 9.33 and 6.30 μM, respectively.

Andrographis paniculata Nees (Acanthaceae), a well-known herbal medicine widely distributed in southeast China, is used extensively as an anti-inflammatory and antipyretic medicine for the treatment of fever, cold, laryngitis, diarrhea, and inflammation.[1] The crude extract of *A. paniculata* and its major *ent*-labdane diterpenoid lactones have been reported to display antiviral,[2] bacteriostatic,[3] immunostimulatory,[4] and hepatoprotective and hepatostimulating[5] activities. The EtOH extract of this plant and its major constituents have been found to affect the cell cycle progression of prostrate and breast cancer cells.[6] Herein we report the structure elucidation of the two pairs of stereoisomers (**1**−**4**) and the antiproliferative activities in human leukemia HL-60 cells of these and the *ent*-labdane diterpenoid derivatives (**5**−**18**).[7]

Compound **1** was obtained as white plates (MeOH), mp 207–208 °C; $[\alpha]^{23}_{D}$ −26.7 (*c* 0.22, MeOH). The IR spectrum showed the presence of hydroxy (3399 cm^{-1}), α,β -unsaturated γ-lactone (1753 cm^{-1}), and *exo*-methylene (914 cm^{-1}) groups. Positive Legal and Kedde color reactions[8] confirmed the existence of the α,β-unsaturated γ-lactone moiety in **1**. The HRESIMS analysis (*m/z* 373.1992 [M + Na]$^+$) in combination with the ^{1}H and ^{13}C NMR data (Table 1) indicated the molecular formula $C_{20}H_{30}O_5$. The characteristic ^{13}C NMR data indicated that **1** was an *ent*-labdane diterpene with an exocyclic methylene at δ 104.2 (C-17), a tertiary methyl at δ 23.8 (C-18), a hydroxymethyl at δ 64.3 (C-19), and an angular methyl group at δ 15.5 (C-20). The NMR spectrum also indicated an α,β-unsaturated γ-lactone moiety with two protons at δ 7.22 (1H, H-14, br s) and 4.74 (2H, H_2-15, br s) and corresponding ^{13}C NMR signals at δ 134.1 (C-13), 145.5 (C-14), 70.7 (C-15), and 174.7 (C-16). The ^{1}H and ^{13}C NMR data were similar to those of the known 14-deoxyandrographolide.[7,9,10] A significant difference in the NMR spectra of the two compounds was observed at C-7, with one signal at δ 4.25 (1H, dd, J = 10.8, 5.4 Hz) assignable to the hydroxymethine (δ 73.6) in **1** instead of two signals at δ 2.36 (1H, dt, J = 12.8, 4.0 Hz) and 1.93 (1H, overlapped) assignable to the methylene (δ 23.7) in 14-deoxyandrographolide. This was further confirmed by the HMBC correlations of H-7 (δ 4.25) with C-6 (δ 34.7), C-8 (δ 151.4), and C-17 (δ 104.2). In the NOESY spectrum, the correlations of H-7 (δ 4.25) with H-5 (δ 1.34), H-6β (δ 1.73), and H-6α (δ 2.50) demonstrated that H-7 was β-oriented, and consequently, 7-OH was in an α-position. Thus, the C-7 configuration of **1** was *R*.[11] On the basis of these evidence, compound **1** was established as 7*R*-hydroxy-14-deoxyandrographolide (Figure 1).

Table 1. ^{1}H and ^{13}C NMR (δ) Data of Compounds **1** and **2** (600 MHz for ^{1}H NMR, 150 MHz for ^{13}C NMR, in pyridine-d_5)

	1		**2**	
position	δ_C	δ_H (J in Hz)	δ_C	δ_H (J in Hz)
1	37.2 t	1.70 m	37.4 t	1.73 br d (12.6)
		1.12 dt (12.6, 5.1)		1.25 dt (12.6, 5.1)
2	29.1 t	2.01 m	29.2 t	2.05 m
		1.98 m		1.97 m
3	79.9 d	3.66 o[a]	80.2 d	3.70 o[a]
4	43.2 s		42.9 s	
5	53.2 d	1.34 dd (12.9, 2.0)	47.9 d	2.20 br d (12.2)
6	34.7 t	2.50 br d (12.9)	32.0 t	2.22 o[a]
		1.73 br d (10.8)		1.67 br d (12.2)
7	73.6 d	4.25 dd (10.8, 5.4)	73.1 d	4.61 br s
8	151.4 s		151.1 s	
9	54.6 d	1.68 br d (10.8)	50.5 d	2.56 br d (10.8)
10	39.2 s		39.7 s	
11	22.3 t	1.82 o[a]	21.9 t	1.80 m
		1.79 o[a]		1.60 m
12	24.8 t	2.57 m	24.6 t	2.52 m
		2.24 m		2.21 o[a]
13	134.1 s		134.1 s	
14	145.5 d	7.22 br s	145.6 d	7.19 br s
15	70.7 t	4.74 br s	70.7 t	4.70 br s
16	174.7 s		174.7 s	
17	104.2 t	5.90 br s	108.4 t	5.14 s
		5.03 br s		4.81 s
18	23.8 q	1.53 s	23.7 q	1.59 s
19	64.3 t	4.47 d (10.8)	64.5 t	4.52 d (10.8)
		3.66 o[a]		3.70 o[a]
20	15.5 q	0.76 s	14.6 q	0.74 s

[a] "o" denotes overlapping signals.

Compound **2** was obtained as white needles (MeOH), mp 225–226 °C; $[\alpha]^{23}_{D}$ +8.3 (*c* 0.20, MeOH). The IR spectrum also showed the presence of hydroxy (3351 cm^{-1}), α,β-unsaturated γ-lactone (1746 cm^{-1}), and *exo*-methylene (917 cm^{-1}) groups. Again, positive Legal and Kedde color reactions[8] confirmed the unsaturated γ-lactone moiety. The HRESIMS analysis (*m/z* 373.1948

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10.1021/np0704452 CCC: $40.75
Published on Web 03/22/2008

Table 2. ^{1}H and ^{13}C NMR (δ) data of Compounds **3** and **4** (600 MHz for ^{1}H NMR, 150 MHz for ^{13}C NMR, in pyridine-d_5)

	3		**4**	
position	δ_C	δ_H (J in Hz)	37.4 t	δ_H (J in Hz)
1	37.4 t	1.85 br d (13.2)	37.4 t	2.04 br d (12.0)
		1.37 m		1.53 dt (12.0, 5.4)
2	29.1 t	2.01 m	29.1 t	2.00 o[a]
		1.97 o[a]		1.97 m
3	80.0 d	3.60 m	79.9 d	3.63 o[a]
4	43.3 s		43.3 s	
5	55.6 d	1.27 br d (12.6)	55.5 d	1.20 br d (12.6)
6	24.7 t	1.81 br d (12.6)	24.7 t	1.78 br d (12.6)
		1.36 m		1.33 m
7	39.1 t	2.36 br d (12.9)	38.7 t	2.32 br d (12.6)
		1.96 o[a]		1.83 dt (12.6, 4.2)
8	148.7 s		148.8 s	
9	51.7 d	2.51 br d (10.8)	53.2 d	1.92 br d (10.8)
10	38.9 s		39.7 s	
11	30.2 t	2.20 br t (12.0)	30.4 t	2.80 br t (12.0)
		2.08 br t (12.0)		2.08 br t (12.0)
12	66.3 d	4.76 d (6.3)	70.6 d	4.55 br d (10.0)
13	51.7 d	2.98 d (6.3)	54.1 d	3.12 br s
14	69.6 d	4.97 br s	73.3 d	5.02 br t (2.4)
15	75.4 t	4.52 br d (10.6)	76.1 t	4.89 t (10.0)
		4.48 d (10.6)		4.50 dd (10.0, 2.4)
16	178.4 s		177.3 s	
17	107.7 t	5.14 br s	107.4 t	4.88 br s
		4.95 br s		4.85 br s
18	23.8 q	1.47 s	23.8 q	1.49 s
19	64.3 t	4.50 d (10.8)	64.3 t	4.43 d (10.5)
		3.60 d (10.8)		3.63 o[a]
20	15.8 q	0.75 s	15.5 q	0.72 s

[a] "o" denotes overlapping signals.

$[M + Na]^+$) indicated the molecular formula $C_{20}H_{30}O_5$ by combining the ^{1}H and ^{13}C NMR data (Table 1). The ^{13}C NMR data of **2** showed characteristic signals similar to those of **1**, including an exocyclic methylene at δ 108.4 (C-17), a tertiary methyl at δ 23.7 (C-18), a hydroxymethyl at δ 64.5 (C-19), an angular methyl group at δ 14.6 (C-20), and an α,β-unsaturated γ-lactone moiety at δ 134.1 (C-13), 145.6 (C-14), 70.7 (C-15), and 174.7 (C-16). The ^{13}C NMR data of **2** were similar to those of **1**, except for the carbon signals at δ 47.9 (C-5), 32.0 (C-6), and 50.5 (C-9), which were shifted upfield by 5.3, 2.7, and 4.1 ppm, respectively, and a signal at δ 108.4 (C-17), which was shifted downfield by 4.2 ppm in **2**. The ^{1}H NMR spectra of the two compounds showed the H-7 resonance at δ 4.61 (1H, brs) in **2** compared to δ 4.25 (1H, dd, J = 10.8, 5.4 Hz) in **1**. These spectroscopic data suggested that **2** was the C-7 epimer of **1**. In the NOESY spectrum, the presence of correlations of H-7 (δ 4.61) with 20-CH_3 (δ 0.74), H-6α (δ 2.22), and H-6β (δ 1.67) and the absence of correlation of H-7 (δ 4.61) with H-5 (δ 2.20) further confirmed the α-orientation of H-7 and the β-position of 7-OH. Thus, the C-7 configuration of **2** was *S*.[11] On the basis of these data, compound **2** was established as 7*S*-hydroxy-14-deoxyandrographolide (Figure 1).

Compound **3** was obtained as a white powder (MeOH), mp 131–132 °C; $[\alpha]^{23}_D$ −4.7 (*c* 0.21, MeOH). The IR spectrum showed the presence of hydroxy (3437 cm^{-1}), lactone carbonyl (1758 cm^{-1}), and *exo*-methylene (915 cm^{-1}) groups. The HRESIMS analysis (*m*/*z* 391.2091 $[M + Na]^+$) and the NMR data (Table 2) revealed the molecular formula $C_{20}H_{32}O_6$. The ^{13}C NMR signals indicated the presence of a labdane-type skeleton with an *exo*-methylene at δ 107.7 (C-17), a tertiary methyl at δ 23.8 (C-18), a hydroxymethyl at δ 64.3 (C-19), and an angular methyl group at δ 15.8 (C-20), which are similar to those of most *ent*-labdane diterpenoids from *A. paniculata*.[7,10] The NMR data of **3** were similar to those of andrographolide,[7,10,12] except for the absence of signals for a trisubstituted C-12−C-13 olefinic bond and the presence of an extra hydroxymethine proton at δ 4.76 (1H, d, J = 6.3 Hz) [δ_C 66.3 (C-12)] and a methine at δ 2.98 (1H, d, J = 6.3 Hz) [δ_C 51.7 (C-13)] in **3**. The HMBC correlations of H-12 (δ 4.76) with C-11 (δ 30.2), C-9 (δ 51.7), and C-16 (δ 178.4) and of H-13 (δ 2.98) with

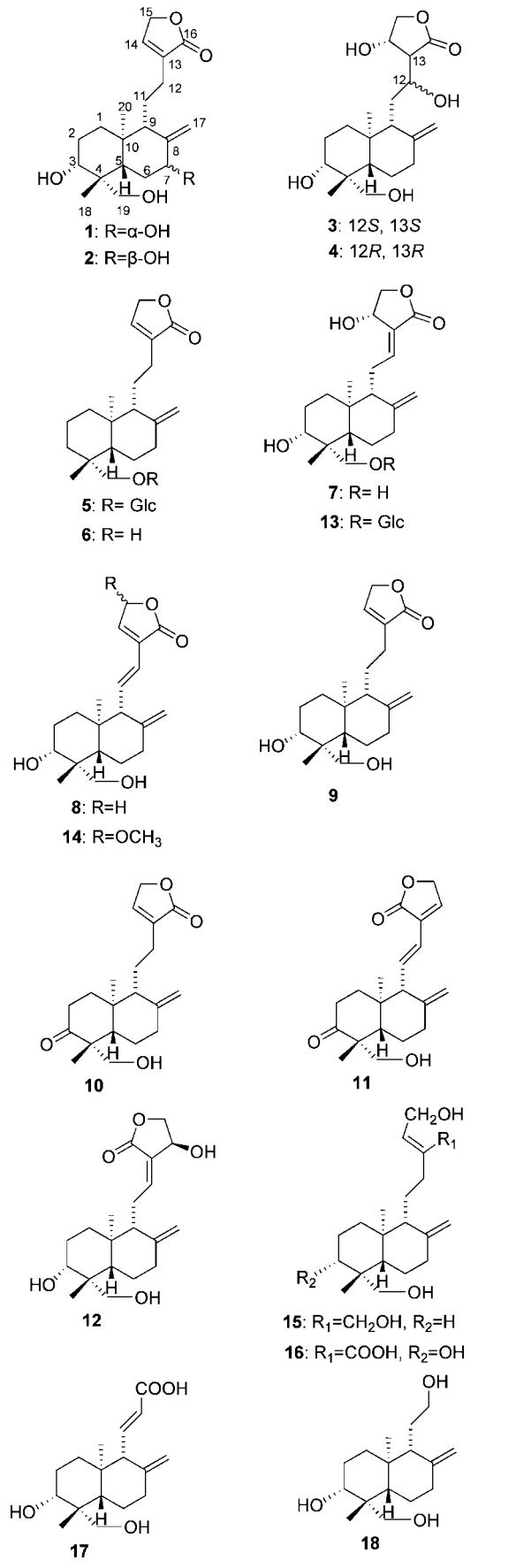

Figure 1. Structures of compounds **1**–**18** from *Andrographis paniculata*.

Table 3. Antiproliferative Effects[a] in HL-60 Cells[b] of Compounds **1**−**18** from *Andrographis paniculata*

compound	$GI_{50} \pm SD$ (μM)	compound	$GI_{50} \pm SD$ (μM)
1	22.48 ± 1.83	**10**	22.80 ± 1.55
2	25.18 ± 1.47	**11**	19.17 ± 2.09
3	24.43 ± 2.19	**12**	6.30 ± 0.65
4	26.56 ± 1.79	**13**	28.83 ± 2.18
5	26.67 ± 0.81	**14**	26.36 ± 1.89
6	20.01 ± 1.22	**15**	20.41 ± 0.73
7	9.33 ± 0.92	**16**	22.42 ± 1.88
8	25.62 ± 1.64	**17**	28.81 ± 1.39
9	25.46 ± 2.13	**18**	24.95 ± 2.10
adriamycin[c]	0.018 ± 0.003		

[a] Data were expressed as mean ± SD of three independent experiments. [b] HL-60 cells were treated with the indicated compounds for 3 days. [c] Adriamycin was used as positive control.

C-16 (δ 178.4), C-15 (δ 75.4), C-14 (δ 69.6), C-12 (δ 66.3), and C-11 (δ 30.2) further supported that the C-12−C-13 double bond in andrographolide was hydroxylated at C-12.

The C-12 configuration could be determined from the chemical shifts of the vinyl protons at C-17.[9,13] Due to the deshielding effect of the hydroxy group at C-12, H_2-17 in the 12*S*-isomer occur more downfield than in the corresponding 12*R*-isomer. Compound **4**, isolated from *A. paniculata*, had identical spectroscopic data with those of a known compound 12*S*-hydroxyandrographolide reported by Shen *et al*.[9] However, the ^{1}H NMR spectra in our study exhibited two H-17 signals at δ 4.88 and 4.85 in **4** (Table 2), and the corresponding protons occurred more downfield at δ 5.14 and 4.95 in **3**. These data suggested that **4** should possess a 12*R*-configuration, and **3** a 12*S*-configuration.[9,13] The configuration of C-13 was not mentioned in the previous report.[9] To determine the C-13 configuration of **3** and **4**, the ^{13}C NMR values were calculated at the B3LYP/6-311+G(2d,p)//3LYP/6-31G(d) level using the GIAO method after a low-energy conformation search using the Hyperchem package.[14,15] The magnetic shielding values were converted into chemical shifts after the corrections using slope and intercept of the linear-square functions.[16,17] The ^{13}C NMR values were calculated for each enantiomer. The relative errors between **3** and **4** are summarized in Tables S1 and S2 of the Supporting Information. In combination with the evidence from the maximum error and the relative error magnitudes, the most likely C-13 configuration of **3** and **4** should be 13*S* and 13*R*, respectively. Thus, compound **3** was elucidated to be 12*S*,13*S*-hydroxyandrographolide, and compound **4** 12*R*,13*R*-hydroxyandrographolide (Figure 1).

The antiproliferative effects of two pairs of stereoisomers (**1**−**4**) and 14 other *ent*-labdane diterpenoid derivatives including neoandrographolide (**5**),[7,9,10,19] 3,14-dideoxyandrographolide (**6**),[7,9,10] andrographolide (**7**),[7,10,12] 14-deoxy-11,12-didehydroandrographolide (**8**),[7,9,10,20] 14-deoxyandrographolide (**9**),[7,9,10] 3-oxo-14-deoxyandrographolide (**10**),[21] 3-oxo-14-deoxy-11,12-didehydroandrographolide (**11**),[7] isoandrographolide (**12**),[7,10,22] andrographiside (**13**),[7,10] 15-methoxy-3,19-dihydroxy-8(17), 11,13-*ent*-labdatrien-16,15-olide (**14**),[7] 8(17),13-*ent*-labdadiene-15,16,19-triol (**15**),[7] 3,15,19-trihydroxy-8(17),13-*ent*-labdadien-16-oic acid (**16**),[7] 3,19-dihydroxy-14,15,16-trinor-8(17),11-*ent*-labdadiene-13-oic acid (**17**),[7] and 3,12,19-trihydroxy-13,14,15,16-tetranor-*ent*-labd-8(17)-ene (**18**)[7] were determined in human leukemia HL-60 cells using a method reported previously.[18]

All these diterpenoid derivatives showed antiproliferative effects (Table 3). Compounds **7** and **12** were the most active, with GI_{50}'s of 9.33 and 6.30 μM, while compounds **14**, **15**, **16**, **17**, and **18** showed weaker activities, with GI_{50}'s of 26.36, 20.41, 22.42, 28.81, and 24.95 μM, respectively. This was attributed to the presence of a hydroxy group on the α,β-unsaturated γ-lactone ring in compounds **7** and **12**. Compounds **3** and **4** showed lower activity than compound **7**, suggesting that the double bond on the lactone ring of **7** plays an augmenting role in the antiproliferative effects. Compound **7** was less effective than **12**, suggesting that the geometrical isomerization (*Z* or *E* form) of the C-12−C-13 double bond influences the antiproliferative effect. The similar activities between compounds **9** and **10** suggest that a carbonyl or a C-3 hydroxymethine does not conspicuously influence the antiproliferative effect. Compounds **5** and **13**, which have a sugar moiety at C-19, showed significantly decreased activities compared with their aglycones **6** and **7**, indicating that a sugar moiety will decrease the antiproliferative effects of these compounds.

Experimental Section

General Experimental Procedures. Melting points were determined with an X-5 hot stage microscope melting point apparatus (uncorrected). Optical rotations were obtained on a P-1020 digital polarimeter (JASCO Corporation). IR spectra were measured with a Bruker IFS 55 spectrometer. NMR spectra were recorded on a Bruker ARX-600 spectrometer (600 MHz for ^{1}H and 150 MHz for ^{13}C) in pyridine-d_5 with TMS as an internal standard. Chemical shifts were expressed in δ (ppm), and coupling constants were reported in *J*. HRESIMS spectra were acquired on a Bruker APEX II mass spectrometer. Preparative HPLC was performed with an ODS column (C-18, 250 × 20 mm, Inertsil Pak) in a Waters 600 liquid chromatograph apparatus equipped with a Waters 490 UV detector. Methanol was HPLC grade. Si gel 60 (Qingdao Haiyang Chemical Co., Ltd., China), Sephadex LH-20 (Advanced Technology Industrial Co., Ltd.), and ODS (40–75 μm, Fuji Silysia Chemical Ltd., Japan) were used as column chromatography stationary phases. TLC was carried out on Si gel 60, and the spots were visualized by spraying with Kedde reagent. All reagents were analytic grade and purchased from Shenyang Chemical Company (Shenyang, China).

Computational Methods. The conformation searches for isomers 13*R*-**3** and 13*S*-**3**, 13*R*-**4**, and 13*S*-**4** were performed with the Hyperchem 7.0 package using the AM1 force field. Geometries, with energies in the window of 0.0–3.0 kcal/mol compared with the most stable conformation, were used for further optimization at the B3LYP/3-21G* level. The geometries with an energy window of 0.0–2.5 kcal/mol were then used in further optimizations at the B3LYP/6-31G* level. Ten totally B3LYP/6-31G*-optimized geometries were used in the ^{13}C NMR calculations at the B3LYP/6-311+G (2d,p) level with the GIAO method for determination of the C-13 configurations in compounds **3** and **4**.

Plant Material. The dried aerial parts of *A. paniculata* were collected from Fujian Province, China. A voucher specimen (AP-2003-824) was identified by Prof. Qi -Shi Sun, and a voucher specimen was deposited at the Department of Natural Products Chemistry, Shenyang Pharmaceutical University, China.

Extraction and Isolation. The plant material (10 kg) was cut into small pieces and heated at reflux with 85% aqueous EtOH (3 × 80 L). The resulting EtOH extract was concentrated *in vacuo*, suspended in H_2O, and partitioned between cyclohexane and EtOAc. The EtOAc extract (295 g) was subjected to Si gel column chromatography (10 cm × 120 cm) using a gradient mixture of $CHCl_3$−MeOH (98:2, 97:3, 95:5, 9:1, 8:2) as eluent to give eight fractions (Fr. 1–8). Fr. 6 was eluted with $CHCl_3$−MeOH (1:1) on Sephadex LH-20. Then the diterpenoid-containing fraction was subjected to a Si gel column by eluting with $CHCl_3$−MeOH (97:3, 95:5, 9:1) and then purified by ODS column chromatography eluted with MeOH−H2O (3:7; 5:5; 7:3) and by repeated RP-18 HPLC preparation to give **1** (31.0 mg), **2** (58.6 mg), **3** (10.6 mg), and **4** (40.4 mg).

7*R*-Hydroxy-14-deoxyandrographolide (1): white plates (MeOH); mp 207–208 °C; $[\alpha]^{23}_D$ −26.7 (*c* 0.22, MeOH); UV (MeOH) λ_{max} 215 nm; IR ν^{KBr}_{max} cm^{-1} 3399, 2967, 2942, 1753, 1651, 1090, 1027, 914; HRESIMS *m/z* 373.1992 $[M + Na]^+$ (calcd for $C_{20}H_{30}O_5Na$, 373.1991); ^{1}H NMR data (600 MHz, pyridine-d_5), see Table 1; ^{13}C NMR data (150 MHz, pyridine-d_5), see Table 1.

7*S*-Hydroxy-14-deoxyandrographolide (2): white needles (MeOH); mp 225–226 °C; $[\alpha]^{23}_D$ +8.3 (*c* 0.22, MeOH); UV (MeOH) λ_{max} 225 nm; IR ν^{KBr}_{max} cm^{-1} 3351, 2943, 1746, 1645, 1445, 1075, 1031, 917; HRESIMS *m/z* 373.1948 $[M + Na]^+$ (calcd for $C_{20}H_{30}O_5Na$, 373.1991); ^{1}H NMR data (600 MHz, pyridine-d_5), see Table 1; ^{13}C NMR data (150 MHz, pyridine-d_5), see Table 1.

12*S*,13*S* -Hydroxyandrographilide (3): white powder (MeOH); mp 131–132 °C; $[\alpha]^{23}_D$ −4.7 (*c* 0.21, MeOH); UV (MeOH) λ_{max} 229 nm; IR ν^{KBr}_{max} cm^{-1} 3437, 2933, 1758, 1649, 1410, 1373, 1169, 1076, 1038, 975, 915; HRESIMS *m/z* 391.2091 $[M + Na]^+$ (calcd. for $C_{20}H_{32}O_6Na$,

391.2097); ^{1}H NMR data (600 MHz, pyridine-d_5), see Table 2; ^{13}C NMR data (150 MHz, pyridine-d_5), see Table 2.

12*R*,13*R*-Hydroxyandrographilide (4): white plates (MeOH); mp 184–185 °C; $[\alpha]^{23}_D$ −62.2 (*c* 0.22, MeOH); UV (MeOH) λ_{max} 202 nm; IR ν^{KBr}_{max} cm^{-1}: 3413, 2929, 1756, 1649, 1457, 1375, 1175, 1079, 1033, 977, 904; HRESIMS *m*/*z* 391.2091 $[M + Na]^+$ (calcd for $C_{20}H_{32}O_6Na$, 391.2097); ^{1}H NMR data (600 MHz, pyridine-d_5), see Table 2; ^{13}C NMR data (150 MHz, pyridine-d_5), see Table 2.

Bioassay. Cells. HL-60 cells were cultured in RPMI-1640 medium supplemented with 100 units/mL penicillin, 100 μg/mL streptomycin, 1mmol/L L-glutamine, and 10% (v/v) heat-inactivated fetal bovine serum.

Antiproliferative Assay. HL-60 cells were seeded at a density of 1 × 10^5 cells/mL and incubated with various concentrations of test compounds (5, 10, 20, 40, 60 μM) for 3 days. Cell growth inhibition was determined by trypan blue staining. The total cell number in each group was counted using a hematocytometer, and the drug concentration that inhibited half of the cell growth (GI_{50}) was calculated. Studies were performed in three independent experiments.

Acknowledgment. This work was supported by the Fund of the Educational Department of Liaoning Province, China (20060882), and partially supported by Joint Research Fund for Overseas Chinese Young Scholars of National Natural Science Foundation of China (30328030).

Supporting Information Available: Computational methods and results. This material is available free of charge via the Internet at http://pubs.acs.org.

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NP0704452