Pregnane alkaloids from *Pachysandra axillaris*

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ABSTRACT

Nine new pregnane alkaloids, pachysamines J–R (**1–9**), together with seven known ones, were isolated from *Pachysandra axillaris*. The chemical structures of the new alkaloids were elucidated by spectroscopic methods. All the compounds were evaluated for their inhibitory activities against HL-60, SMMC-7721, A-549, SK-BR-3, and PANC-1 cell lines. Compound **15** possessed moderate activities against A-549, SK-BR-3, and PANC-1 cells, with the IC_{50} values of 11.17, 4.17, and 10.76 μM, respectively. Besides, compound **11** showed cytotoxicities against A-549 cell, with the IC_{50} values as 24.94 μM.

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1. Introduction

The plants of *Pachysandra* genus (Buxaceae) are widely distributed in south of China. And *Pachysandra axillaris*, as a kind of evergreen subshrub, is a popular plant in Yunnan and Sichuan Provinces. Some *Pachysandra* species, such as *P. axillaris*, have been used as the folk medicine for the treatment of pain and stomach trouble, and their fruits are edible [1]. Previously a series of pachysandra alkaloids, which belong to pregnane alkaloid derivatives, were isolated from this genus [2–8]. The compounds, pachysandrine A and pachystermine A, isolated initially from *Pachysandra terminalis*, possess anti-ulcer effect [9–11]. And compounds pachysanonin, spiropachysine, and pachyaximine A, previously obtained from *P. axillaris*, were reported to have anticancer activities [8,12]. For our interest in the anticancer bioactivities of pachysandra alkaloids, we further reinvestigated *P. axillaris* and nine new pregnane alkaloids, pachysamines J–R (**1–9**), together with seven known analogues, pachysamine B (**10**), pachysamine H (**11**), axillarine C (**12**), vaganine A (**13**), pachysanonin (**14**), sarcovagine D (**15**), and axillaridine A (**16**), were isolated. Herein, we describe the isolation and structural elucidation of the new pregnane alkaloids and their cytotoxic evaluation.

2. Experimental

2.1. General methods

Optical rotations were measured with a Horiba SEPA-300 polarimeter. IR spectra were recorded on a Bio-Rad FTS-135 infrared spectrophotometer. 1D and 2D NMR spectra experiments were measured in $CDCl_3$ on Bruker AM-400 and (or) DRX-500 instruments, and chemical shifts (δ) were expressed in ppm with reference to the solvent signals. MS data were obtained on a VG Autospec-3000 mass spectrometer (the used matrix material was glycerol). Column chromatography was performed on silica gel (200–300 mesh, Qingdao Marine Chemical Inc., Qingdao, People's Republic of China), alumina (Jinshan Works, Shanghai, People's Republic of China), and Sephadex LH-20 (Pharmacia). TLC was performed with glass precoated Si gel GF_{245} plates (Qingdao Marine Chemical Inc., Qingdao, People's Republic of China). Solvents used for extraction and isolation were distilled prior to use.

2.2. Plant material

The plants of *P. axillaris* were collected in the environs of Kunming City, Yunnan Province, People's Republic of China, in August 2007. The sample was identified by Prof. Xi-Wen Li at Kunming Institute of Botany, Chinese Academy of Sciences.

2.3. Extraction and isolation

The air-dried and powdered plants of *P. axillaris* (69 kg) were extracted with 95% EtOH, and the crude extract was adjusted

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Table 1
^{13}C NMR spectral data of compounds **1–9**[a].

No.	1	2	3	4	5	6	7	8	9
1	47.8	44.4	39.1	39.9	38.9	37.3	36.7	33.0	32.7
2	71.9	71.4	114.7	126.2	125.6	28.5	27.1	24.9	24.9
3	55.0	53.5	130.4	131.5	131.5	49.1	56.3	60.1	60.7
4	34.0	75.8	72.3	196.6	196.2	35.4	75.1	25.9	25.9
5	45.0	49.8	45.5	54.8	53.0	45.3	52.6	42.7	42.7
6	32.0	25.7	24.0	20.5	20.4	28.9	22.6	72.1	72.1
7	31.6	32.0	31.6	30.6	30.6	31.9	31.5	37.0	37.0
8	34.6	34.9	35.3	34.7	34.0	35.4	35.0	32.1	32.1
9	56.2	56.8	54.3	54.0	53.9	54.1	54.2	54.9	54.9
10	36.7	34.8	33.8	40.0	39.9	35.4	36.7	38.9	38.8
11	21.1	20.4	20.7	20.8	20.5	21.0	20.9	72.5	71.7
12	39.5	39.5	39.5	39.1	39.9	39.7	39.6	82.2	82.3
13	42.7	41.7	41.5	41.7	41.6	42.0	41.6	47.7	47.6
14	56.6	56.4	56.3	56.3	54.7	56.4	56.5	52.6	52.4
15	24.0	24.0	24.0	23.9	34.6	24.0	24.0	24.1	24.0
16	27.3	27.6	27.6	27.6	72.4	27.6	27.6	26.1	26.1
17	54.0	54.7	54.7	54.9	58.9	54.4	54.7	60.7	60.1
18	12.2	12.4	12.1	12.3	14.2	12.2	12.3	9.7	9.7
19	13.1	17.5	13.8	13.3	13.3	12.3	13.6	13.3	13.3
20	62.2	61.1	61.1	61.1	56.7	61.9	61.1	209.1	209.2
21	12.2	9.8	9.8	9.9	9.8	10.3	9.8	30.8	30.8
NMe$_2$	39.5	39.9	39.8	39.9	39.9	39.7	39.8	43.5	43.5
1′	169.0	167.1	167.9	165.7	165.5	165.0	173.1	171.3	171.4
2′	118.0	134.3	132.2	118.7	118.6	121.1	42.4	38.9	40.4
3′	151.9	128.6	131.1	153.7	153.1	140.6	122.1	133.3	120.1
4′	27.2	127.1	12.1	27.5	27.4	134.9	130.5	128.6	128.9
5′	19.8	131.6	14.1	19.7	19.8	128.7	20.7	129.5	20.6
6′		127.1				127.7	20.7	127.1	20.6
7′		128.6				128.5	19.2	129.5	19.4
8′						127.7		128.6	
9′						128.7			
Ac-C=O			173.7					170.9	171.2
Ac-Me			21.0					21.2	21.2
Ac-C=O								170.8	170.8
Ac-Me								20.7	20.9

[a] Spectra were recorded in CDCl$_3$; chemical shifts (δ) in ppm.

to pH 2 with 3% HCl. The acid-soluble fraction was alkalinized to pH 9 with 1% NaOH followed by exhaustive extraction with CHCl$_3$. CHCl$_3$-soluble materials were roughly separated by silica gel column chromatography, using CHCl$_3$/MeOH (from 1:0 to 0:1) as eluent, to give five fractions (Fr. A1–A5). Fr. A1 was chromatographed over an alumina column with a mixture of petroleum ether/acetone (from 1:0 to 3:1) and a silica gel column with a mixture of petroleum ether/acetone/Et$_2$NH (from 100:5:2 to 100:30:10) followed by Sephadex LH-20 column chromatography eluted with MeOH to afford **1** (6 mg), **2** (12 mg), **3** (27 mg), and **15** (21 mg). Fr. A2 eluted with petroleum ether/acetone/Et$_2$NH (from 100:10:2 to 100:40:10) was separated by silica gel column chromatography to yield **3** (45 mg), **11** (39 mg), and **12** (8 mg). Fr. A3 was further chromatographed over an alumina column with petroleum ether/acetone/Et$_2$NH (from 100:10:2 to 100:50:10) and then was eluted on the silica gel column with petroleum ether/acetone/Et$_2$NH (from 100:10:2 to 100:50:10) repeatedly to provide **5** (40 mg), **7** (44 mg), and **8** (12 mg). Fr. A4 was also chromatographed over the alumina column with petroleum ether/acetone/Et$_2$NH (from 100:20:4 to 100:60:10) and the silica gel column with petroleum ether/acetone/Et$_2$NH (from 100:20:4 to 100:60:15), to yield **9** (25 mg), **10** (15 mg), **13** (9 mg), **14** (38 mg), and **16** (11 mg). Fr. A5 was chromatographed by silica gel with CHCl$_3$/MeOH (from 1:0 to 3:1) column and Sephadex LH-20 column (MeOH) to give **3** (36 mg), **4** (9 mg), and **6** (15 mg).

2.3.1. Pachysamine J (**1**)

C$_{28}$H$_{48}$N$_2$O$_2$; white powder; $[\alpha]_D^{19.6}$ +22.4 (c 0.83, CHCl$_3$); IR (KBr) ν_{max} 3429, 2931, 2847, 1637, 1525, 1445 cm^{-1}; positive FABMS m/z [M+H]$^+$ 445 (100), 72 (34); positive HRESIMS m/z 445.3790 [M+H]$^+$ (calc for C$_{28}$H$_{49}$N$_2$O$_2$ [M+H]$^+$, 445.3794); ^{1}H and ^{13}C NMR data, see Tables 1 and 2.

2.3.2. Pachysamine K (**2**)

C$_{30}$H$_{46}$N$_2$O$_3$; white needles; $[\alpha]_D^{27.8}$ +13.6 (c 0.95, CHCl$_3$); IR (KBr) ν_{max} 3431, 2930, 2849, 1639, 1523, 1486 cm^{-1}; positive FABMS m/z [M+H]$^+$ 483 (36), 111 (23); 97 (48); 83 (57); 72 (89); positive HRESIMS m/z 483.3581 [M+H]$^+$ (calc for C$_{30}$H$_{47}$N$_2$O$_3$ [M+H]$^+$, 483.3587); ^{1}H and ^{13}C NMR data, see Tables 1 and 2.

2.3.3. Pachysamine L (**3**)

C$_{30}$H$_{48}$N$_2$O$_3$; white crystal; $[\alpha]_D^{27.6}$ +95.0 (c 0.95, CHCl$_3$); IR (KBr) ν_{max} 3360, 2970, 2864, 1717, 1678, 1640, 1535, 1444, 1370 cm^{-1}; positive FABMS m/z [M+H]$^+$ 485 (28); 425 (12); 72 (100); positive HRESIMS m/z 485.3739 [M+H]$^+$ (calc for C$_{30}$H$_{49}$N$_2$O$_3$ [M+H]$^+$, 485.3743); ^{1}H and ^{13}C NMR data, see Tables 1 and 2.

2.3.4. Pachysamine M (**4**)

C$_{28}$H$_{44}$N$_2$O$_2$; white powder; $[\alpha]_D^{27.8}$ +11.5 (c 0.77, CHCl$_3$); IR (KBr) ν_{max} 3511, 2928, 1664, 1523 cm^{-1}; positive FABMS m/z [M+H]$^+$ 441 (37); positive HRESIMS m/z 441.3467 [M+H]$^+$ (calc for C$_{28}$H$_{45}$N$_2$O$_2$ [M+H]$^+$, 441.3481); ^{1}H and ^{13}C NMR data, see Tables 1 and 2.

2.3.5. Pachysamine N (**5**)

C$_{28}$H$_{44}$N$_2$O$_3$; white crystal; $[\alpha]_D^{27.3}$ +28.7 (c 1.08, CHCl$_3$); IR (KBr) ν_{max} 3384, 3287, 2989, 2940, 1667, 1640, 1516, 1448, 1368, 1336 cm^{-1}; positive FABMS m/z [M+H]$^+$ 457 (76); 83 (51); 72 (100); positive HRESIMS m/z 457.3423 [M+H]$^+$ (calc for C$_{28}$H$_{45}$N$_2$O$_3$ [M+H]$^+$, 457.3430); ^{1}H and ^{13}C NMR data, see Tables 1 and 2.

Table 2
^{1}H NMR spectral data of compounds **1–5**[a].

No.	1	2	3	4	5
1	1.98–2.18b; 0.88–1.00b	2.10–2.21b; 1.25–1.30b	2.16–2.25b; 1.83–1.95b	2.26–2.59b; 2.17–2.36b	2.45–2.58b; 2.22–2.36b
2	3.46 (m)	4.23 (m)	6.82 (m)	7.82 (m)	7.66 (m)
3	3.58 (m)	4.07 (m)			
4	1.51–1.56b; 1.32–1.35b	3.99 (m)	5.10 (m)		
5	1.13–1.25b	1.18–1.3b	1.64–1.71b	2.22–2.32b	2.20–2.29b
6	1.69–1.86b; 0.88–0.98b	1.74–1.88b; 1.27–1.38b;	2.05–2.12b; 1.31–1.42b	2.00–2.06b; 1.21–1.43b	2.00–2.10b; 1.26–1.48b
7	1.57–1.64b; 0.80–0.88b	1.70–0.81b; 0.90–0.98b	1.73–1.81b; 0.88–0.96b	1.77–1.87b; 0.81–0.96b	1.82–1.98b; 0.82–0.95b
8	1.42–1.65b	1.33–1.46b	1.29–1.40b	1.23–1.38b	1.18–1.31b
9	0.94–1.11b	0.57–0.68	0.69–0.81b	0.94–1.05b	0.85–0.96b
11	1.46–1.50b; 1.18–1.33b	1.38–1.48b; 1.23–1.37b	1.65–1.72b; 1.42–1.58b	1.46–1.58b; 1.21–1.43b	1.48–1.58b; 1.26–1.48b
12	1.79–1.89b; 1.02–1.17b	1.81–1.93b; 1.03–1.14b	1.14–1.22b; 1.09–1.20b	2.17–2.30b; 1.10–1.22b	1.81–1.97b; 1.05–1.18b
14	0.94–1.11b	0.94–1.10b	0.98–1.09b	1.00–1.12b	0.95–1.08b
15	1.34–1.44b; 1.23–1.33b	1.51–1.62b; 0.99–1.10b	1.56–1.70b; 1.03–1.09b	1.38–1.51b; 1.03–1.13b	1.81–1.99b; 0.82–0.97b
16	1.71–1.83b; 1.23–1.33b	1.66–1.88b; 1.33–1.48b;	1.39–1.48b; 1.82–1.91b	1.84–1.96b; 1.46–1.58b	4.31 (m)
17	1.29–1.40b	1.26–1.33b	1.35–1.43b	1.37–1.47b	1.18–1.31b
18	0.75 (s)	0.66 (s)	0.65 (s)	0.66 (s)	0.88 (s)
19	1.21 (s)	1.25 (s)	1.04 (s)	0.86 (s)	0.86 (s)
20	2.38 (m)	2.40 (m)	2.42 (m)	2.62 (m)	2.93 (m)
21	0.61 (d, 7.5)	0.86 (d, 6.4)	0.87 (d, 6.4)	1.07 (d, 6.5)	0.90 (d, 6.4)
N-Me$_2$	2.79 (s)	2.16 (s)	2.17 (s)	2.17 (s)	2.23 (s)
2′	5.56. s			5.66 (s)	5.65 (s)
3′		7.44 (m)	6.42 (m)		
4′	1.79 (s)	7.84 (m)	1.76 (d, 7.07)	1.78 (s)	1.87 (s)
5′	2.08 (s)	7.52 (m)	2.09 (s)	1.88 (s)	2.17 (s)
6′		7.84 (m)			
7′		7.44 (m)			
Ac-Me			1.82 (s)		

[a] Spectra were recorded in CDCl$_3$; chemical shifts (δ) in ppm with J values in Hz.
[b] Overlapped.

2.3.6. Pachysamine O (6)

C$_{32}$H$_{48}$N$_2$O; white crystal; $[\alpha]_D^{27.7}$ −14.7 (c 1.10, CHCl$_3$); IR (KBr) ν_{max} 3261, 2930, 2851, 1658, 1621, 1538, 1447, 1384, 1340 cm^{-1}; positive FABMS m/z [M+H]$^+$ 477 (22); 72 (100); positive HRESIMS m/z 477.3831 [M+H]$^+$ (calc for C$_{32}$H$_{49}$N$_2$O [M+H]$^+$, 477.3845); ^{1}H and ^{13}C NMR data, see Tables 1 and 3.

2.3.7. Pachysamine P (7)

C$_{30}$H$_{52}$N$_2$O$_2$; white needles; $[\alpha]_D^{27.8}$ −25.9 (c 0.90, CHCl$_3$); IR (KBr) ν_{max} 3431, 3349, 2936, 2866, 1610, 1539, 1444, 1382 cm^{-1}; positive FABMS m/z [M+H]$^+$ 473 (100); 72 (36); positive HRESIMS m/z 473.4098 [M+H]$^+$ (calc for C$_{30}$H$_{53}$N$_2$O$_2$ [M+H]$^+$, 473.4107); ^{1}H and ^{13}C NMR data, see Tables 1 and 3.

Table 3
^{1}H NMR spectral data of compounds **6–9**[a].

No.	6	7	8	9
1	1.69–1.78b; 1.02–1.13b	1.65–1.72b; 1.04–1.10b	1.28–1.49b; 1.09–1.18b	1.57–1.65b; 1.05–1.15b
2	1.83–1.96b; 1.17–1.29b	1.71–1.80b; 1.33–1.44b	1.67–1.81b; 1.27–1.40b	1.66–1.71b; 1.41–1.52b
3	3.89 (m)	3.61 (m)	1.90–2.04b	1.95–1.06b
4	1.61–1.72b; 1.30–1.37b	3.17 (m)	1.72–1.87b; 1.08–1.23b	1.71–1.95b; 1.14–1.26b
5	1.17–1.30b	1.03–1.13b	1.69–1.84b	1.74–1.86b
6	1.84–1.96b; 1.29–1.43b	1.95–2.05b; 1.06–1.17b	4.55 (m)	4.58 (m)
7	1.61–1.72b; 0.85–0.96b	1.69–1.78b; 0.79–0.90b	1.97–2.08b	1.98–2.09b;
8	1.13–1.27b	1.29–1.41b	1.30–1.50b	1.63–1.75b
9	0.64–0.74b	0.65–0.76b	1.39–1.54b	1.40–1.54b
11	1.48–1.57b; 1.21–1.33b	1.42–1.51b; 1.21–1.32b	5.28 (dd, 10.4, 9.0)	5.28 (m)
12	1.83–1.93b; 1.11–1.21b	1.84–1.92b; 1.08–1.17b	4.74 (d, 9.0)	4.74 (d, 8.8)
14	1.00–1.13b	0.99–1.10b	1.27–1.40b	1.29–1.43b
15	1.57–1.69b; 1.04–1.17b	1.35–1.44b; 1.00–1.12b	1.66–1.82b; 1.26–1.41b	1.70–1.83; 1.31–1.43b
16	1.89–2.00b; 1.29–1.43b	1.81–1.89b; 1.39–1.50b	1.96–2.08b; 1.72–1.87b	1.98–2.10b; 1.71–1.95b
17	1.35–1.46b	1.31–1.41b	2.71 (m)	2.72 (m)
18	0.64 (s)	0.62 (s)	0.92 (s)	0.94 (s)
19	0.80 (s)	0.80 (s)	0.98 (s)	1.01 (s)
20	2.41 (m)	2.39 (m)		
21	0.86 (d, 6.3)	0.84 (d, 6.3)	2.15 (s)	
N-Me$_2$	2.17 (s)	2.15 (s)	2.17 (s)	2.20 (s)
2′	6.35 (d, 15.6)	3.02 (s)	3.46 (m)	2.91 (s)
3′	7.60 (d, 15.6)			
4′			7.20–7.31b	
5′	7.35 (m)	1.69 (s)	7.20–7.31b	1.64 (s)
6′	7.48 (m)	1.69 (s)	7.20–7.31b	1.64 (s)
7′	7.42 (m)	1.74 (s)	7.20–7.31b	1.67 (s)
8′	7.48 (m)		7.20–7.31b	
9′	7.35 (m)			
Ac-Me			2.01 (s)	2.02 (s)
Ac-Me			1.98 (s)	2.00 (s)

[a] Spectra were recorded in CDCl$_3$; chemical shifts (δ) in ppm with J values in Hz.
[b] Overlapped.
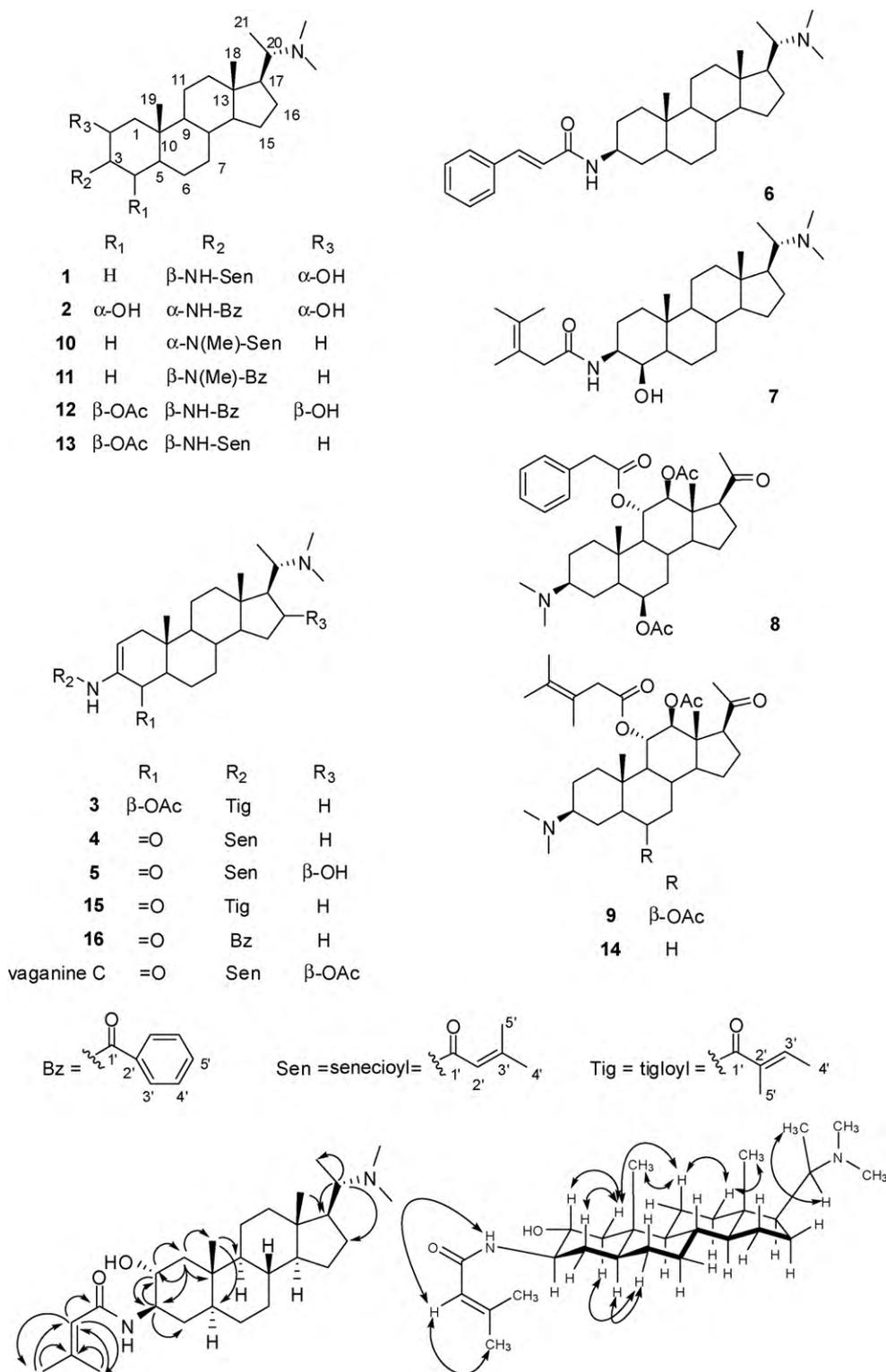
Fig. 1. Key HMBC (→) and ROESY (↔) correlations of 1.

2.3.8. Pachysamine Q (8)

$C_{35}H_{49}NO_7$; white crystal (MeCOMe); $[\alpha]_D^{27.5}$ +44.2 (c 0.93, CHCl$_3$); IR (KBr) ν_{max} 3487, 2973, 1737, 1456, 1369 cm^{-1}; positive ESIMS m/z [M+H]$^+$ 596 (100); positive HRESIMS m/z 596.3597 [M+H]$^+$ (calc for $C_{35}H_{50}NO_7$ [M+H]$^+$, 596.3587); ^{1}H and ^{13}C NMR data, see Tables 1 and 3.

2.3.9. Pachysamine R (9)

$C_{34}H_{53}NO_7$; white crystal; $[\alpha]_D^{27.5}$ +54.5 (c 1.04, CHCl$_3$); IR (KBr) ν_{max} 3474, 2978, 2766, 1726, 1460, 1374, 1246 cm^{-1}; positive FABMS m/z [M+H]$^+$ 588 (100); positive HRESIMS m/z 588.3901 [M+H]$^+$ (calc for $C_{34}H_{54}NO_7$ [M+H]$^+$, 588.3900); ^{1}H and ^{13}C NMR data, see Tables 1 and 3.
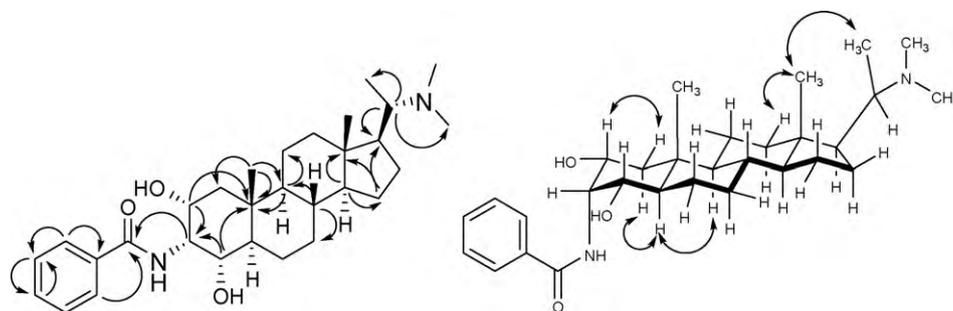
Fig. 2. Key HMBC(→) and ROESY (↔) correlations of **2**.

2.4. Cytotoxicity testing

Cytotoxicity of compounds against suspended tumor cells was determined by the MTT method. All the cells were cultured in RPMI-1640 or DMEM medium (Hyclone, USA), supplemented with 10% fetal bovine serum (Hyclone, USA) at 37 °C in a humidified atmosphere with 5% CO_2.

Cell viability was assessed by conducting colorimetric measurements of the amount of insoluble formazan formed in living cells based on the reduction of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT). Briefly, 100 μL of adherent cells was seeded into each well of 96-well cell culture plates and allowed to adhere for 12 h before drug addition, while suspended cells were seeded just before drug addition, both with initial density of 1×105 cells/ml in 100 μl medium. Each tumor cell line was exposed to the tested compound at various concentrations in triplicates for 48 h, with cisplatin as positive control. After the incubation, MTT (100 μg) was added to each well, and the incubation continued for 4 h at 37 °C. The cells were lysed with 100 μl 20% SDS–50% DMF after removal of 100 μl medium. The optical density of the lysate was measured at 595 nm in a 96-well microtiter plate reader. Cell growth inhibition curve was graphed and IC_{50} value of each compound was calculated by the Reed and Muench's method [13].

3. Results and discussion

An EtOH extract of *P. axillaris* was treated with aqueous HCl to give a crude alkaloid fraction. The alkaloid fraction yielded 16 pachysandra alkaloids by repeated silica gel, alumina, Sephadex LH-20 chromatography, and recrytallization. Besides compounds **1**–**9**, compounds **10**–**16** were elucidated to be pachysamine B [14], pachysamine H [6], axillarine C [15], vaganine A [16], pachysanonin [8], sarcovagine D [17], and axillaridine A [18], separately by comparison the ^{1}H NMR and ^{13}C NMR spectral data.

Pachysamine J (**1**) was obtained as white powder. Its molecular formula, $C_{28}H_{48}N_2O_2$, was established on the basis of HRESIMS

analysis ([M+H]+, *m/z* 445.3790). The ^{1}H NMR spectrum showed characteristic signals at δ_H 0.75 (3H, s, Me-18), 1.21 (3H, s, Me-19), 0.61 (3H, d, *J* = 6.6, Me-21), 2.79 (6H, s, Me$_2$N). In addition, the ^{13}C NMR and DEPT experiments showed signals for seven methyls, eight methylenes, nine methines (including one oxygenated at δ_C 71.9 and one olefinic carbon at δ_C 118.0), and four quaternary carbons (including one olefinic carbon at δ_C 151.9 and a carbonyl one at δ_C 169.0). Moreover, considering the abundance of pregnane alkaloids in the *Pachysandra* genus, **1** was proposed to have a basic skeleton of 20-(dimethylamino) pregnane. The positive FABMS exhibited a diagnostic fragment of N-ethylidene-N-dimethylaminium at *m/z* 72 (33%), which also suggested a 20-(dimethylamino) pregnane skeleton [19]. Comparison of the spectroscopic data of **1** and **10** revealed similarities except for the absence of a methyl on the nitrogen at C-3 and the presence of a hydroxyl group at C-2 in **1**. This was supported by the downfield chemical shift of C-1 (δ_C 47.8), C-3 (δ_C 55.0), and C-5 (δ_C 45.0) in **1**, compared with those of C-1 (δ_C 35.6), C-3 (δ_C 39.8), and C-5 (δ_C 41.7) in **10**. HMBC correlations of H-2 (δ_H 3.46) with C-1 (δ_C 47.8) and C-3 (δ_C 55.0) further supported the assignment. Besides, for the α-orientation of 2-OH group was determined by the ROESY correlations of H-1β with H-2 (Fig. 1). The other substituents had the same orientations as those in **10**. Therefore, **1** was elucidated as 20α-dimethylamino-3β-senecioylamino-5α-pregnane-2α-ol.

Pachysamine K (**2**) had the molecular formula $C_{30}H_{46}N_2O_3$, as determined by HRESIMS analysis ([M+H]+, *m/z* 483.3581). The IR spectrum of **2** showed a hydroxyl characteristic absorption band at 3431 cm^{-1}. And the positive FABMS also exhibited a diagnostic fragment at *m/z* 72 (88%), which suggested a 20-(dimethylamino) pregnane [19]. The ^{1}H NMR spectrum displayed five methyl signals at δ_H 0.66, 1.25, 0.86, 2.16, and 2.16. In the ^{13}C NMR spectrum, signals for five methyls (δ_C 12.4, 17.5, 9.8, 39.9, and 39.9), seven methylenes (δ_C 44.4, 25.7, 32.0, 20.4, 39.5, 24.0, and 27.6), fourteen methines (δ_C 71.4, 53.5, 75.8, 49.8, 34.9, 56.8, 56.4, 54.7, 61.1, 128.6, 127.1, 131.6, 127.1, and 128.6), and four quaternary carbons (δ_C 34.8, 41.7, 167.1, and 134.3) were observed. Analysis of the spectra revealed the 20-(dimethylamino) pregnane. The NMR
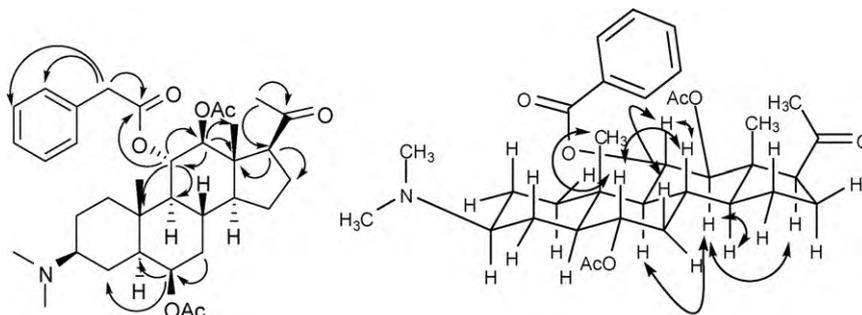

Fig. 3. Key HMBC(→) and ROESY (↔) correlations of **8**.

Table 4
^{13}C NMR spectral data of compounds **10–16**[a].

No.	**10**	**11**	**12**	**13**	**14**	**15**	**16**
1	35.6t	35.5t	44.4t	37.2t	32.7t	39.6t	39.5t
2	24.0t	24.8t	67.7d	24.3t	25.1t	126.4t	126.7d
3	39.8d	40.2d	52.8d	49.6d	61.0d	132.3s	131.5s
4	32.8t	31.7t	72.8d	75.3d	32.1t	196.5s	196.4s
5	41.7d	41.6d	48.5d	48.9d	33.8d	55.2d	54.9d
6	28.8t	28.7t	27.2t	25.3t	28.9t	20.5t	20.5t
7	31.8t	29.1t	31.8t	31.8t	31.6t	30.9t	30.8t
8	35.5d	35.3d	34.9d	35.1d	38.0d	35.0d	34.7d
9	54.7d	54.3d	56.2d	56.1d	55.5d	54.3d	54.0d
10	35.2s	34.9s	34.7s	35.5s	40.5s	39.6s	40.1s
11	20.8t	20.7t	20.2t	20.4t	72.1d	20.8t	20.8t
12	39.8t	39.6t	39.6t	39.4t	82.6d	40.3t	39.1t
13	41.7s	41.9s	41.7s	41.7s	47.7s	42.2s	41.7s
14	56.6d	56.4d	56.4d	56.1d	52.8d	56.4d	56.3d
15	24.0t	23.9t	23.5t	24.2t	24.2t	24.5t	24.0t
16	27.6t	27.4t	27.8t	27.5t	26.2t	28.0t	27.6t
17	55.0d	54.9d	54.2d	54.7d	60.8d	54.2d	54.9d
18	12.3q	12.2q	12.2q	12.3q	9.7q	12.7q	12.3q
19	12.6q	12.6q	16.4q	14.1q	12.1q	13.7q	13.4q
20	61.2d	61.7d	61.1d	61.3d	209.5s	63.1d	61.1d
21	9.9q	10.2q	9.9q	9.6q	30.8q	10.5q	9.9q
NMe$_2$	39.8q	39.7q	39.87q	39.4q	43.8q	39.8q	39.9q
NMe	26.0q	35.6q					
1′	168.8s	171.7s	167.2s	166.2s	171.6s	168.1s	165.7s
2′	119.5d	137.5s	134.5s	118.4d	38.9t	132.5d	134.7s
3′	143.9s	128.3d	128.6d	151.1s	120.3s	131.8s	128.7d
4′	12.3q	126.4d	127.1d	27.1q	128.7s	14.6q	127.0d
5′	20.1q	129.0d	131.2d	19.7q	20.6q	12.6q	131.8d
6′		126.4d	127.1d		19.7q		127.0d
7′		128.3d	128.6d		20.6q		128.7d
Ac-C=O			169.1s	170.7s	171.2s		
Ac-Me			21.2q	21.1q	21.0q		

[a] Spectra were recorded in CDCl$_3$; chemical shifts (δ) in ppm.

data indicated that the resonances of **2** were very similar to those of **12** except for the replacement of an acetoxy group in **2** by a hydroxyl group at C-4 in **12** [2]. This was supported by the HRES-IMS and HMBC spectrum. The α-orientated hydroxyl groups at C-2 and C-4, and the α-orientated nitrogen group at C-3 was determined from the ROESY correlations of H-1β with H-2β, H-3β, H-4β and H-1α with H-5α, H-9α (Fig. 2). The other substituents had the same orientations as those in **12**. Thus, **2** was elucidated as 20α-dimethylamino-3α-benzoylamino-5α-pregnane-2α,4α-diol.

Pachysamine L (**3**) had the same molecular formula (C$_{30}$H$_{48}$N$_2$O$_3$) as vaganine C [2]. Its positive FABMS fragment (*m/z* 72) and NMR data suggested **3** also to be a 20-(dimethylamino) pregnane alkaloid [19]. Its NMR spectra were similar to those of vaganine C except for the signals of the functional group at C-3. The senecioyl group in vaganine C was replaced by a tigloyl one, which was confirmed by the HMBC experiments. In this spectrum, long-range correlations observed from H-3′ (δ_H 6.42, 1H, m) to C-1′ (δ_C 167.9, s), C-4′ (δ_C 12.1, q), and C-5′ (δ_C 14.1, q). Therefore, the structure of **3** was represented as 20α-dimethylamino-4β-acetoxy-3-tigloylamino-Δ^2-5α-pregnane.

The molecular formula of pachysamine M (**4**) was inferred as C$_{28}$H$_{44}$N$_2$O$_2$ by HRESIMS and NMR data. The IR data showed absorption of conjugated ketone group at 1664 cm^{-1}. Comparison the spectroscopic data of **4** and **16** revealed similarities except for the replacement of a benzoyl group on the nitrogen atom at C-3 in **16** by a senecioyl functional group in **4** [2]. HMBC correlations of the resonances at H-4′ (δ_H 1.78, 3H, s) with C-1′ (δ_C 165.7), H-5′ (δ_H 1.88, 3H, s) with C-1′ (δ_C 165.7), and H-2′ (δ_H 7.82, 1H, m) with C-1′ (δ_C 165.7) further supported the assignment. So **4** was characterized as 20α-dimethylamino-3-senecioylamino-5α-pregn-2-en-4-one.

Pachysamine N (**5**) has the molecular formula C$_{28}$H$_{44}$N$_2$O$_3$, as determined by HRESIMS. Comparison of the spectroscopic data of **5** and **4** revealed similarities except for one more hydroxyl group at

C-16 in **4**, which in according with the downfield chemical shift of C-15 (δ_C 34.6), C-16 (δ_C 72.4), and C-17 (δ_C 58.9), and upfield shift of C-14 (δ_C 53.0), and C-20 (δ_C 56.7). HMBC correlations of H-16 (δ_H 4.31, 1H, m) with C-13 (δ_C 41.6), C-14 (δ_C 53.0), and C-15 (δ_C 34.6) further supported the assignment. The β-orientation of the 16-OH group was deduced from the correlations of H-16/H-17 in ROESY. Therefore, **5** was elucidated as 20α-dimethylamino-16β-hydroxy-3-senecioylamino-5α-pregn-2-en-4-one.

Pachysamine O (**6**) was assigned the molecular formula C$_{32}$H$_{48}$N$_2$O by positive HRESIMS. The characteristic fragment ion of 20-(dimethylamino) pregnane at *m/z* 72 (100%) was observed in the positive FABMS [19]. The ^{1}H NMR spectrum of **6** showed signals for a *trans*-double bond [δ_H 6.35 (1H, d, *J* = 15.6), and 7.60 (1H, d, *J* = 15.6)], as well as five aromatic protons [δ_H 7.35 (2H, m), 7.42 (1H, m), and 7.48 (2H, m)]. Analysis of the ^{13}C NMR spectrum indicated the presence of a *trans*-double bond (δ_C 121.1, and 140.6), and a benzene ring signals (δ_C 134.9, 128.7, 127.7, 128.5, 127.7, and 128.7). There were no palpable differences in the NMR spectrum between **6** and **11**, except for the absence of a methyl on the nitrogen at C-3 in **6** and the replacement of the benzoyl group in **11** by a cinnamoyl one on the nitrogen at C-3 in **6** [2]. The HMBC correlations observed from H-3′ (δ_H 7.60, 1H, d, *J* = 15.6) to C-4′ (δ_C 134.9), C-5′ (δ_C 128.7), and C-9′ (δ_C, 128.7) gave the cinnamoyl group. Besides, in HMBC correlations of H-2′ (δ_H 6.35, 1H, d, *J* = 15.6) with C-1′ (δ_C 165.0), of H-3′ (δ_H 7.60, 1H, d, *J* = 15.6) with C-1′ (δ_C 165.0), and of H-3 (δ_H 3.89, 1H, m) with C-1′ (δ_C 165.0), also supported this group was linked to a carbonyl carbon at C-3. Thus, **6** was identified as 20α-dimethylamino-3β-cinnamoylamino-5α-pregnane.

The ^{1}H and ^{13}C NMR data of pachysamine P (**7**) were very similar to those of **13**. The differences were that the acetoxyl group at C-4 and the senecioyl group on the nitrogen at C-3 in **13** were replaced by a hydroxyl at C-4 and a 3,4-dimethylpent-3-enoyl group on the nitrogen at C-3 in **7**, respectively [2]. In NMR spectra, the HMBC

Table 5
Cytotoxic activities of compounds **11**, **13**, and **15**[a].

Compound	HL-60	SMMC-7721	A-549	SK-BR-3	PANC-1
15	2.96	16.69	11.17	4.17	10.76
13	24.22	>40	>40	>40	>40
11	13.80	>40	24.94	>40	>40
Cisplatin	1.51	11.45	25.69	10.32	24.44

[a] Results are expressed as IC$_{50}$ values in μM.

correlations from the ^{1}H NMR signals of H-3 (δ_H 3.61, 1H, m) to the ^{13}C NMR signals of ketone C-1' (δ_C 173.1), C-2 (δ_C 27.1), and C-4 (δ_C 75.1), suggested the above assignment. According to the correlations of H-4/H-5 in ROESY, the 4-OH was identified as β-oriented. Eventually, **7** was elucidated as 20α-dimethylamino-3β-(3,4-dimethylpent-3-enoyl)amino-5α-pregnane-4β-ol.

Pachysamine Q (**8**) had the molecular formula C$_{35}$H$_{49}$NO$_7$, as determined by HRESIMS. Comparison the spectroscopic data of **8** and **14** revealed similarities except for an additional acetoxyl group at C-6 in **8** and the replacement of 3,4-dimethylpent-3-enoyl group in **14** by a phenylacetyl group in **8** [8]. The acetylation at C-6 caused the downfield chemical shift of C-6 (δ_C 72.1) and the upfield chemical shift of C-5 (δ_C 42.7) and C-7 (δ_C 37.0). The observed HMBC correlations of H-6 (δ_H 4.55, 1H, m) with C-4 (δ_C 25.9), C-5 (δ_C 42.7), and carbonyl carbon (δ_C 170.8) at C-6 further supported the above assignment (Fig. 3). ROESY cross-peaks for H-6/H-19, H-6/H-8, H-12/H-9, and H-12/H-17 indicated that 6-OAc was α-oriented and 12-OAc was β-oriented. And the phenylacetyl group was placed at C-11, as established by the HMBC spectrum. Hence, the structure of 6α,12β-diacetoxy-3β-(dimethylamino)-11α-phenylaceoxypregnan-20-one was assigned to **8**.

Pachysamine R (**9**) gave a molecular formula of C$_{34}$H$_{53}$NO$_7$ by HRESIMS. The NMR data indicated that **9** was very similar to **8** except that the phenylacetyl group at C-11 in **8** was replaced by a 3,4-dimethylpent-3-enoyl group in **9**. The 3,4-dimethylpent-3-enoyl group was placed at C-11, as established by the HMBC spectrum. All the substituents had the same orientations as those in **8**, which were proved by the ROESY. Consequently, **9** was characterized as 6β,12β-diacetoxy-3β-(dimethylamino)-11α-[(3,4-dimethylpent-3-enoyl)oxy] pregnan-20-one.

The structures of known compound **10–16** were elucidated by their spectral data. And their ^{13}C NMR spectral data are shown Table 4.

The cytotoxicities of all the compounds were tested against breast cancer SK-BR-3, hepatocellular carcinoma SMMC7721, human myeloid leukemia HL-60, pancreatic cancer PANC-1, and lung cancer A549 cell lines. Compound **15** was cytotoxic for all the test cell lines, with the IC$_{50}$ value of 2.96, 16.69, 11.17, 4.17, 10.76 μM, respectively. Besides, compounds **11** and **13** showed selective cytotoxicity against some of the cells (Table 5). And the other compounds showed no inhibitory activity against the tumor cells tested with IC$_{50}$ values greater than 40 μM.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.steroids.2010.05.005.

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