FULL PAPER

Three New Pregnane Alkaloids from *Pachysandra terminalis*

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Three new pregnane alkaloids, pachystermine C (**1**), pachysanamine A (**2**), and pachysanamine B (**3**), together with four known ones, pachystermine B (**4**), pachysamine A (**5**), (20*S*)-20-(dimethylamino)-16α-hydroxy-3*β*-(3′α-isopropyl)lactam-5α-pregnan-4-one (**6**), and *E*-salignone (**7**), were isolated from *Pachysandra terminalis*. 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, MCF-7, and SW480 cell lines, some of the compounds showed stronger cytotoxicity for the test cell lines, especially compounds **2**, **3**, and **7**.

Keywords: Pregnane alkaloids, Buxaceae, *Pachysandra terminalis*, Cytotoxicity.

Introduction

Pachysandra is a genus of evergreen perennials or subshrubs, belonging to the boxwood family Buxaceae, and *Pachysandra terminalis* (common names Japanese pachysandra), is native to Japan, Korea, and P. R. China [1][2]. A series of chemical study of *Pachysandra* genus has been carried out, which led to the isolation of many pregnane alkaloids. In particular, some of them had shown antitumor and antiulcer activities [3 – 12]. It is known that the habitat has a strong impact on the secondary metabolites of the plants. Though many phytochemical studies on the plants of *Pachysandra* genus had been carried out, there was no report on the *P. terminalis* which grows in P. R. China. And in this investigation, three new pregnane alkaloids (**1** – **3**), together with four known ones, pachystermine B (**4**) [4][13], pachysteramine A (**5**) [4][13], (20*S*)-20-(dimethylamino)-16α-hydroxy-3*β*-(3′α-isopropyl)lactam-5α-pregnan-4-one (**6**) [14], and *E*-salignone (**7**) [15], were isolated from *P. terminalis* growing in P. R. China (*Fig. 1*). Herein, the structural characterization of compounds **1** – **3** and their cytotoxicities were given.

Results and Discussion

Pachystermine C (**1**) was obtained as a white powder, for which the molecular formula was assigned as $C_{29}H_{50}N_2O_3$ on the basis of the HR-EI-MS (*m*/*z* 474.3812, $[M]^+$). And the positive FAB-MS exhibited a diagnostic fragment of

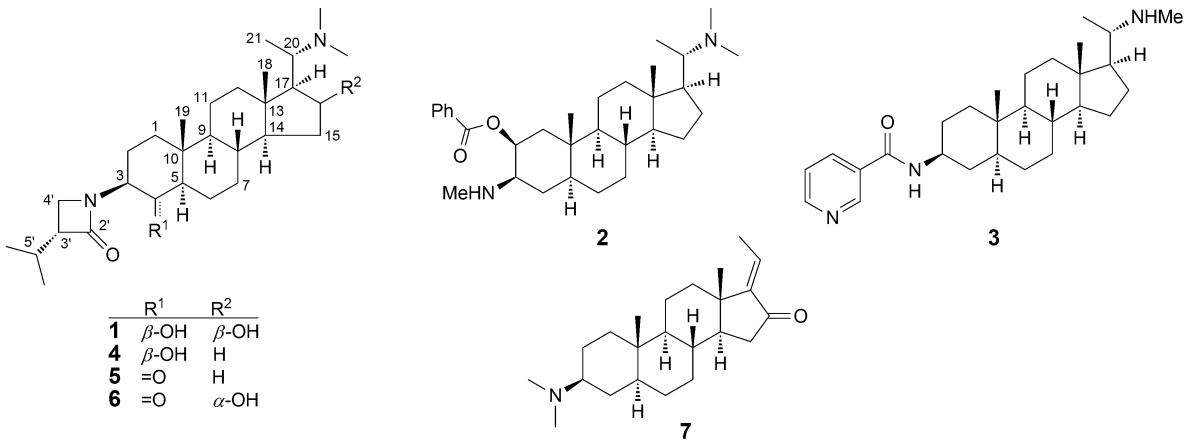

Fig. 1. Structures of compounds **1** – **7**.

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N-ethylidene-*N*-dimethylaminium at *m*/*z* 72 (100%), which suggested a 20-(dimethylamino) pregnane skeleton [16] . The ^{1}H-NMR spectrum (*Table 1*) showed characteristic signals: δ(H) 0.86 (3H, *s*, Me(18)), 1.07 (3H, *s*, Me (19)), 0.92 (3H, *d*, *J* = 6.5 Hz, Me(21)), 2.23 (6H, *s*, Me_2(N)). In addition, ^{13}C-DEPT data (*Table 1*) showed signals for seven methyls, eight methylenes, eleven methines (including two oxygenated: δ(C) 72.3 (*d*) and δ(C) 75.5 (*d*)), and four quaternary carbons (including a carbonyl one: δ(C) 170.0 (*s*)). Considering the abundance of pregnane alkaloids in the *Pachysandra* genus, compound **1** was proposed to have a basic skeleton of 20-(dimethylamino)pregnane.

A comparison of the molecular formula of **1** and **4** revealed that there was an O-atom more in **1** than **4**. The spectroscopic data of **1** and **4** were similar, and the only difference was that **1** had one more OH group. The additional OH group was positioned at C(16) due to the signals shifted downfield to δ(C) 72.5 (C(16) in **1** from δ(C) 27.6 C(16) in **4**, and δ(C) 34.7 (C(15) in **1** from δ(C) 24.0 C(15) in **4**. In the HMBC spectrum (*Fig. 2*), the following signal correlations were observed: H–C(16) (δ(H) 4.30 (*dd*, J = 13.8, 7.6)) with C(13), H–C(15) (δ(H) 2.14 – 2.20 (*m*)) with C(13), C(14), and C(16), H–C(5) (δ(H) 1.08 – 1.14 (*m*)) with C(4), H–C(3) (δ(H) 3.17 (*dt*, *J* = 14.0, 6.0)) with C(2′) and C(4′), and these confirmed

Table 1. ^{1}H- and ^{13}C-NMR data of compounds **1** – **3**. δ in ppm, *J* in Hz.

Position	**1**		**2**		**3**	
	δ(H)	δ(C)	δ(H)	δ(C)	δ(H)	δ(C)
1	1.71 – 1.78 (*m*), 0.89 – 0.99 (*m*)	37.4 (*t*)	1.81 – 1.87 (*m*), 1.54 – 1.63 (*m*)	38.2 (*t*)	1.68 – 1.73 (*m*), 0.92 – 0.99 (*m*)	37.3 (*t*)
2	1.75 – 1.83 (*m*), 1.32 – 1.36 (*m*)	25.8 (*t*)	5.32 (*dt*, *J* = 12.3, 4.4)	73.9 (*d*)	1.77 – 1.87 (*m*), 1.48 – 1.57 (*m*)	26.8 (*t*)
3	3.17 (*dt*, *J* = 14.0, 6.0)	58.9 (*d*)	3.02 – 3.08 (*m*)	57.8 (*d*)	4.19 – 4.25 (*m*)	48.7 (*d*)
4	4.04 (*m*)	72.3 (*d*)	1.42 – 1.51 (*m*), 1.14 – 1.33 (*m*)	27.7 (*t*)	1.49 – 1.54 (*m*), 1.04 – 1.07 (*m*)	35.1 (*t*)
5	1.08 – 1.14 (*m*)	49.1 (*d*)	1.54 – 1.63 (*m*)	38.2 (*d*)	1.04 – 1.17 (*m*)	45.2 (*d*)
6	1.36 – 1.43 (*m*), 1.23 – 1.36 (*m*)	20.2 (*t*)	1.54 – 1.65 (*m*), 1.43 – 1.54 (*m*)	30.7 (*t*)	1.73 – 1.79 (*m*), 1.16 – 1.27 (*m*)	28.7 (*t*)
7	1.76 – 1.83 (*m*), 0.82 – 0.96 (*m*)	32.4 (*t*)	1.64 – 1.72 (*m*), 0.88 – 1.02 (*m*)	31.7 (*t*)	1.63 – 1.68 (*m*), 1.46 – 1.54 (*m*)	32.0 (*t*)
8	1.44 – 1.55 (*m*)	34.8 (*d*)	1.54 – 1.63 (*m*)	34.7 (*d*)	1.33 – 1.42 (*m*)	35.3 (*d*)
9	0.55 – 0.59 (*m*)	54.5 (*d*)	0.83 – 0.93 (*m*)	54.1 (*d*)	0.65 – 0.73 (*m*)	54.3 (*d*)
10		35.9 (*s*)		37.3 (*s*)		36.0 (*s*)
11	1.86 – 1.99 (*m*), 1.47 – 1.56 (*m*)	21.5 (*t*)	1.47 – 1.55 (*m*), 1.22 – 1.33 (*m*)	20.9 (*t*)	1.24 – 1.32 (*m*), 1.13 – 1.19 (*m*)	21.6 (*t*)
12	1.77 – 1.86 (*m*), 1.00 – 1.07 (*m*)	40.2 (*t*)	1.84 – 1.93 (*m*), 1.02 – 1.13 (*m*)	39.6 (*t*)	1.85 – 1.97 (*m*), 1.12 – 1.20 (*m*)	39.3 (*t*)
13		41.7 (*s*)		41.6 (*s*)		42.2 (*s*)
14	0.81 – 0.93 (*m*)	53.4 (*d*)	0.98 – 1.09 (*m*)	56.5 (*d*)	1.01 – 1.20 (*m*)	56.8 (*d*)
15	2.14 – 2.20 (*m*), 1.18 – 1.26 (*m*)	34.7 (*t*)	1.53 – 1.63 (*m*), 1.01 – 1.11 (*m*)	24.0 (*t*)	1.53 – 1.63 (*m*), 1.11 – 1.18 (*m*)	23.9 (*t*)
16	4.30 (*dd*, *J* = 13.8, 7.6)	72.5 (*d*)	1.78 – 1.91 (*m*), 1.14 – 1.33 (*m*)	27.6 (*t*)	1.73 – 1.79 (*m*), 1.19 – 1.28 (*m*)	28.4 (*t*)
17	1.19 – 1.25 (*m*)	55.4 (*d*)	1.32 – 1.43 (*m*)	54.7 (*d*)	1.39 – 1.48 (*m*)	56.5 (*d*)
18	0.86 (*s*)	14.7 (*q*)	0.64 (*s*)	12.3 (*q*)	0.75 (*s*)	12.2 (*q*)
19	1.07 (*s*)	14.3 (*q*)	0.93 (*s*)	12.7 (*q*)	0.78 (*s*)	12.3 (*q*)
20	2.89 – 2.96 (*m*)	59.7 (*d*)	2.35 – 2.42 (*m*)	61.1 (*d*)	2.49 – 2.55 (*m*)	59.0 (*d*)
21	0.92 (*d*, *J* = 6.5)	9.8 (*q*)	0.85 (*d*, *J* = 7.5)	9.7 (*q*)	1.27 (*d*, *J* = 6.7)	19.4 (*q*)
Me^1	2.23 (*s*)	40.2 (*q*)	2.16 (*s*)	39.8 (*q*)	2.41 (*s*)	33.0 (*q*)
Me^2			2.41 (*s*)	34.8 (*q*)		
1′				165.7 (*s*)		164.4 (*s*)
2′		170.0 (*s*)		130.5 (*s*)		130.8 (*s*)
3′	2.89 – 2.95 (*m*)	56.7 (*d*)	8.03 (*dd*, *J* = 7.4, 1.1)	129.5 (*d*)	8.90 (*s*)	147.5 (*d*)
4′	3.40 (*t*, *J* = 8.8), 2.89 – 2.95 (*m*)	42.6 (*t*)	7.45 (*t*, *J* = 7.4)	128.4 (*d*)		
5′	1.92 – 1.97 (*m*)	28.0 (*d*)	7.57 (*t*, *J* = 7.4)	132.9 (*d*)	8.70 (*d*, *J* = 4.6)	152.0 (*d*)
Me^3	0.95 (*d*, *J* = 6.7)	19.9 (*q*)				
	1.05 (*d*, *J* = 6.7)	19.8 (*q*)				
6′					7.39 (*dd*, *J* = 7.5, 4.6)	123.5 (*d*)
7′					8.08 (*d*, *J* = 7.5)	135.1 (*d*)
OH	3.71 (*d*, *J* = 3.3, HO–C(4))					
NH					6.02 (*d*, *J* = 8.0, HN–C(3))	

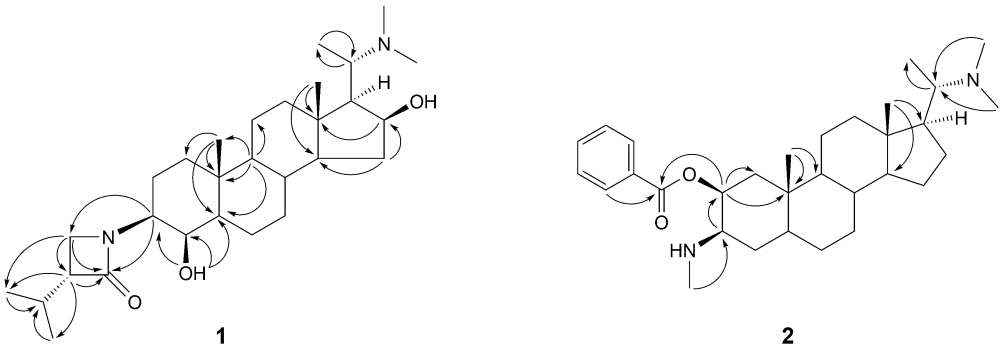

Fig. 2. Key HMBCs (H → C) of **1** and **2**.

the above assignment. The HMBC data of δ(H) 3.71 (*d*, J = 3.3) with C(3), C(4), and C(5) were observed, which proved that the δ(H) 3.71 (*d*, J = 3.3) was the ^{1}H-NMR signal of the HO group at C(4). The ROESY correlations of Hα–C(16) with Hα–C(17), Hα–C(15), and Hα–C(3) with Hα–C(4), Hα–C(5), suggested that the substituents at C(3), C(4), and C(16) all had β-orientations. Therefore, compound **1** was elucidated as (20*S*)-20-(dimethylamino)-4β,16β-dihydroxy-3β-(3′α-isopropyl)lactam-5α-pregnane.

Pachysanamine A (**2**) was isolated as white powder. The molecular formula was determined to be $C_{31}H_{48}N_2O_2$ by HR-EI-MS (*m*/*z* 480.3715, $[M]^+$). And the positive FAB-MS also exhibited a diagnostic fragment at *m*/*z* 72 (100%), which suggested a 20-(dimethylamino) pregnane [16]. The ^{1}H-NMR spectra (*Table 1*) displayed the presence of six Me signals: δ(H) 0.64 (3 H, *s*, Me–C(18)), 0.93 (3 H, *s*, Me–C(19)), 0.85 (3 H, *d*, J = 7.5 Hz, Me–C(21)), 2.41 (3 H, *s*, Me(N)–C(3)), 2.23 (6 H, *s*, Me_2(N)–C(20)).

Careful comparison of ^{1}H- and ^{13}C-NMR data of **2** (*Table 1*) and pachysamine J [17] revealed that the two compounds have the similar skeleton except for the substituent group at N–C(3) and HO–C(2). The senecioyl group at N–C(3) in pachysamine J was replaced by a methyl group, while the H-atom of HO–C(2) was replaced by a benzoyl group, which was confirmed by the HMBC experiments (*Fig. 2*). In the HMBC spectrum, the long-range correlations were observed from H–C(2) (δ(H) 5.32 (*dt*, J = 12.3, 4.4 Hz)) to C(1), C(10) and C(1′), from H–C(1) (δ(H) 1.81 – 1.87 (*m*), 1.54 – 1.63 (*m*)) to C(2), C(10), and from Me–N(C(3)) (δ(H) 2.41 (*s*)) to C(3). The relative configurations of HO–C(2) and C(3) were assigned as β-orientation by correlations of Hα–C(2) with Hα–C(3), and Hα–C(3) with Hα–C(2), Hα–C(5). So, compound **2** was characterized as (20*S*)-(dimethylamino)-3β-*N*-methylamino-2β-benzoyloxy-5α-pregnane.

Pachysanamine B (**3**) was obtained as white powder. Its molecular formula $C_{28}H_{43}N_3O$, determined from the HR-EI-MS, had a CH_2-group less than that of *epi*-pachysamine B [4]. The ^{1}H-NMR spectrum of **3** (*Table 1*) showed four Me signals: δ(H): 0.75 (3 H, *s*, Me–C(18)), 0.78 (3 H, *s*, Me–C(19)), 1.27 (3 H, *d*, J = 6.7 Hz, Me–C (21)), 2.41 (3 H, *s*, Me(N)–C(20)), which were characteristic signals of a pregnane skeleton. Analysis of the ^{13}C-NMR spectrum (*Table 1*) indicated the presence of pyridine ring: δ(C): 130.8 (*s*, C(2′)), 152.0 (*d*, C(3′)), 147.5 (*d*, C(5′)), 123.5 (*d*, C(6′)), 135.1 (*d*, C(7′)). And there were no palpable differences in the NMR spectrum between **3** and *epi*-pachysamine B, except for a Me group less at N–C(20) in **3** than *epi*-pachysamine B. Moreover, the HMBC correlations of **3** (*Fig. 3*) were observed from H–C(2) (δ(H) 1.77 – 1.87 (*m*), 1.48 – 1.57 (*m*)) to C(3), from H–C(3) (δ(H) 4.19 – 4.25 (*m*)) to C(1′). The HMBC correlations of δ(H) 6.02 (*d*, J = 8.0) with C(3) and C(3) were observed, which showed that the δ(H) 6.02 (*d*, J = 8.0) was the ^{1}H-NMR signal of amide NH proton at C(3). Consequently, the structure of **3** was elucidated as (20*S*)-(methylamino)-3β-pyridinecarbonylamino-5α-pregnane.

Compounds **1** – **7** (purity > 90%) were tested for their cytotoxic activities *in vitro* against HL-60, SMMC7721, A549, MCF-7, and SW-480 cell lines (*Table 2*), using the improved MTT method as previously described [17]. Compared with positive control cisplatin (DDP; *Sigma*, St. Louis, USA, purity > 98%), compound **3** has obvious cytotoxicity against all the cell lines with the IC_{50} value of 2.4 ± 0.3, 7.3 ± 0.8, 3.6 ± 0.3, 3.1 ± 0.3, and 3.7 ± 0.4 μM, respectively. Compound **2** showed moderate cytotoxicity against all the cell lines with the IC_{50} value of 3.8 ± 0.5, 15.7 ± 1.4, 10.7 ± 0.5, 13.9 ± 0.8, and 11.4 ± 0.6 μM, respectively. Compound **7** showed selective cytotoxicity against A-549, and MCF-7 cell lines with the

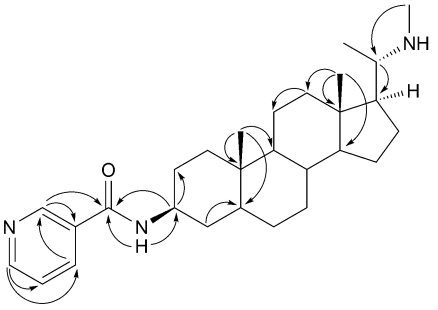

Fig. 3. Key HMBCs (H → C) of **3**.

Table 2. Cytotoxicity of compounds **1** – **7** toward different cancer cells[a])

Compound	HL-60	SMMC-7721	A-549	MCF-7	SW480
1	18.8 ± 1.8	28.1 ± 2.2	15.7 ± 0.8	15.3 ± 0.8	14.0 ± 1.2
2	3.8 ± 0.5	15.7 ± 1.4	10.7 ± 0.5	13.9 ± 0.8	11.4 ± 0.6
3	2.4 ± 0.3	7.3 ± 0.7	3.6 ± 0.3	3.1 ± 0.3	3.7 ± 0.4
4	16.0 ± 0.8	36.9 ± 3.3	17.1 ± 1.3	17.4 ± 1.1	17.2 ± 1.2
5	14.3 ± 0.7	35.2 ± 2.9	19.1 ± 1.4	15.9 ± 1.3	16.6 ± 1.0
6	14.3 ± 0.5	24.3 ± 2.1	17.1 ± 1.3	15.7 ± 1.2	12.9 ± 1.0
7	5.5 ± 0.3	15.2 ± 0.6	6.3 ± 0.7	4.1 ± 0.4	9.4 ± 1.0
Cisplatin	1.0 ± 0.2	14.8 ± 0.7	13.6 ± 0.9	17.1 ± 1.3	15.6 ± 1.5

[a]) Results are expressed as IC_{50} values in μM (mean ± SD, n = 3).

IC_{50} value of 6.3 ± 0.7, 4.1 ± 0.4. The other compounds showed low inhibitory activity against the tumor cells.

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Experimental Part

General

Solvents used for extraction and isolation were distilled prior to use. TLC: precoated silica gel GF_{245} glass plates (*Qingdao Marine Chemical Inc.*, Qingdao, P. R. China). Column chromatography (CC): silica gel (200 – 300 mesh, *Qingdao Marine Chemical Inc.*), alumina (*Jinshan Works*, Shanghai, P. R. China), and *Sephadex LH-20* (*Pharmacia*, Uppsala, Sweden). Optical rotations: *Horiba SEPA-300* polarimeter. IR Spectra: *Bio-Rad FTS-135* infrared spectrophotometer (Berkeley, USA); $\tilde{\nu}$ in cm^{-1}. 1D- and 2D-NMR spectra: *Bruker AV-400*, *DRX-500*, and/or *AV-600* instruments (Billerica, USA) in $CDCl_3$;δ in ppm rel. to the solvent signals, J in Hz. MS: *Autospec Premier P776* mass spectrometer (Washington, D.C., USA; the used matrix material was glycerol); in m/z (rel. %).

Plant Material

The whole plants of *P. terminalis* were collected at Nanjing City, Jiangsu Province of P. R. China, in March 2009. The plant material was identified by Prof. *Xi-Wen Li* and a voucher (No. KIB 20090503d) has been deposited with the State Key Laboratory of Phytochemistry and Plant Resources in West China, Kunming Institute of Botany, Chinese Academy of Sciences.

Extraction and Isolation

Air-dried roots of *P. terminalis* (2 kg) were extracted three times with MeOH. After removal of the solvent under reduced pressure, the residue was obtained. This residue was dissolved in H_2O and adjusted to pH 2 with 3% HCl. The acid-soluble fraction was alkalinized to pH 9 with 5% NaOH followed by exhaustive extraction (5 ×) with $CHCl_3$. $CHCl_3$-soluble material (50 g) was roughly separated by CC on SiO_2 ($CHCl_3$/MeOH 1:0 → 0:1) to give four fractions, *Frs. A1 – A4*. *Fr. A1* was chromatographed over an alumina column with a mixture of petroleum ether (PE)/acetone (1:0 → 4:1) and a silica gel column with a mixture of PE/acetone/Et_2NH (80:2:1 → 20:2:1) followed by *Sephadex LH-20* CC eluted with MeOH to afford **1** (58 mg) and **4** (12 mg). *Fr. A2* eluted with PE/acetone/Et_2NH (100:5:1 → 100:40:10) was separated by CC on SiO_2 and *LH-20* eluted with MeOH to yield **2** (5 mg) and **5** (120 mg). *Fr. A3* was chromatographed over the alumina column with PE/acetone (100:10 → 100:60) and SiO_2 with PE/acetone/Et_2NH (100:20:4 → 100:60:15) to yield **6** (14 mg). *Fr. A4* was also chromatographed over the alumina column with PE/acetone/Et_2NH (100:20:4 → 100:60:10) and SiO_2 with PE/acetone/Et_2NH (100:20:4 → 100:60:15) to yield **3** (37 mg) and **7** (45 mg).

Pachystermine C (= **(3*R*)-1-[(3*β*,4*β*,5*α*, 16*β*,20*S*)-20-(Dimethylamino)-4,16-dihydroxypregnan-3-yl]-3-(1-methylethyl)-azetidin-2-one**; **1**). White powder ($CHCl_3$). $[\alpha]_D^{20}$ = −37.2 (c = 0.75, MeOH). IR (KBr): 3216, 2933, 1708, 1508. ^{1}H- and ^{13}C-NMR: see *Table 1*. FAB-MS (pos.): 475 (62, $[M + H]^+$), 72 (100). HR-EI-MS: 474.3812 (M^+, $C_{29}H_{50}N_2O_3^+$; calc. 474.3821).

Pachysanamine A (= **(2*β*,3*β*,5*α*,20*S*)-20-(Dimethylamino)-3-(methylamino)pregnan-2-yl Benzoate**; **2**). White powder ($CHCl_3$). $[\alpha]_D^{20}$ = −81.7 (c = 0.55, MeOH). IR (KBr): 3456, 2967, 1711, 1506. ^{1}H- and ^{13}C-NMR: see *Table 1*. FAB-MS (pos.): 481 (88, $[M + H]^+$), 359 (10), 314 (9), 72 (100). HR-EI-MS: 480.3715 (M^+, $C_{31}H_{48}N_2O_2^+$; calc. 480.3716).

Pachysanamine B (= ***N*-[(3*β*,5*α*,20*S*)-20-(Methylamino)pregnan-3-yl]pyridine-3-carboxamide**; **3**). White powder ($CHCl_3$). $[\alpha]_D^{20}$ = −24.7 (c = 1.07, MeOH). IR (KBr): 3305, 2928, 1656, 1545, 1458. ^{1}H- and ^{13}C-NMR: see *Table 1*. FAB-MS (pos.): 481 (88, $[M + H]^+$), 359 (10), 314 (9), 72 (100). HR-EI-MS: 437.3414 (M^+, $C_{28}H_{43}N_3O^+$; calc. 437.3406).

Cytotoxicity Tests

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

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