Two New Pregnanone Derivatives with Strong Cytotoxic Activity from *Pachysandra axillaris*

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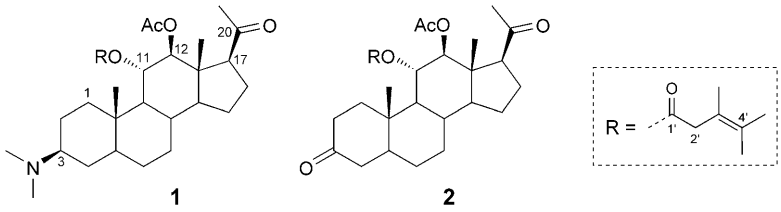
Two new, bioactive, pregnane-based natural products, pachysanonin (= 3β,11α,12β)-12-acetoxy-3-(dimethylamino)-11-[(3,4-dimethylpent-3-enoyl)oxy]pregnan-20-one; **1**) and pachysanone (=(11α,12β)-12-acetoxy-11-[(3,4-dimethylpent-3-enoyl)oxy]pregnan-3,20-dion; **2**) have been isolated from *Pachysandra axillaris*. Their structures were determined by spectroscopic methods, and, in the case of **2**, by single-crystal X-ray crystallography (*Figure*). Compound **2** showed significant antitumor activity against *Lewis* lung carcinoma (LCC) tumor cells, with an IC_{50} value of 0.020 ± 0.006 μg/ml, which is equal or even lower than those of the well-known natural antitumor agents harringtonine (0.02), homoharringtonine (0.15), and adriamycin (0.06 μg/ml; positive control).

1. Introduction. – *Pachysandra axillaris* FRANCH. (*Buxaceae*) is distributed in Southern China and has been used as a folk medicine for the treatment of pain and stomach trouble [1]. Great interest has been generated in *P. axillaris* because of the abundance of its alkaloidal constituents [2–5]. In continuation of our study of secondary metabolites of this plant, we previously reported the isolation and structures of two new alkaloids, paxillarines A and B [6]. Further investigation of bioactive compounds from this plant have now led to the isolation of the steroidal alkaloid pachysanonin (**1**) and the pregnane derivative pachysanone (**2**), whose structure elucidations and bioactivities are reported herein.



2. Results and Discussion. – 2.1. *Chemistry.* Air-dried whole plants (45 kg) of *P. axillaris* were extracted with 95% EtOH. Then, the extract was treated with aqueous AcOH to give a crude alkaloid fraction, which was separated into strongly and weakly basic subfractions. The weakly basic alkaloid fraction was subjected to repeated

purification by column chromatography on alumina and NH_2-silica gel to afford compounds **1** (150 mg) and **2** (30 mg).

Pachysanonin (**1**) had the molecular formula $C_{32}H_{51}NO_5$, as determined by EI-MS, DEPT-NMR, and HR-EI-MS. The mass spectrum exhibited molecular-ion peaks at *m/z* 530 ($[M + H]^+$) and 529 (M^+), and the presence of two intense, diagnostic fragments at *m/z* 110 (97%) and 84 (100%) suggested a 3-(dimethylamino)pregnane skeleton. EI-MS Experiments also indicated $C_6H_{11}COO$ and acetyl (Ac) substituents by characteristic fragments at *m/z* 471($[M + H-AcOH]^+$), 402 ($[M - C_6H_{11}COO]^+$), and 342 ($[M + H-AcOH-C_6H_{11}COO]^+$), respectively.

The IR spectrum of **1** revealed characteristic absorptions for ketone and ester C=O groups at ν_{max} 1745, 1735, 1710, 1270, and 1240 cm^{-1}. The ^{1}H-NMR spectrum showed characteristic signals at δ(H) 0.950 (*s*, Me(18)), 0.959 (*s*, Me(19)), 1.930 (*s*, Me(21)), and 2.210 (*s*, Me_2N). ^{13}C-NMR and DEPT experiments showed signals for nine Me, eight CH_2, and eight sp^3 CH groups, including two oxygenated CH groups at δ(C) 72.2 and 82.6, as well as seven quaternary C-atoms, including two sp^2-hybridized C-atoms at δ(C) 120.3 and 128.8, and three C=O resonances at δ(C) 209.6, 171.2, and 171.6, respectively. Based on our earlier study of the ^{13}C-NMR chemical shifts of *Pachysandra* alkaloids [7], the ^{13}C-NMR data of the 3-(dimethylamino)pregnane skeleton could be readily assigned, as shown in the *Table*. The chemical shifts of the ring-*A* resonances indicate that **1** has a 3β-(Me_2N) group.

HMQC and HMBC Experiments with **1** showed correlations between the signals at δ(H) 4.748 (*d*, $J = 9.6$ Hz, H−C(12)) and δ(C) 171.2 (C=O of Ac), 82.6 (C(12)), and 47.8 (C(13)), and between δ(H) 2.007 (Me of Ac) and δ(C) 171.2 (C=O of Ac), which suggested a β-AcO group in 12-position. By the same method, the HMBC correlations between the signals at δ(H) 5.292 (*dd*, $J = 9.6$ Hz, H−C(11)) and δ(C) 171.6 (O=C(1′)), 72.2 (H−C(11)), and 55.5 (H−C(9)), and those between δ(H) 2.936/2.899 (A_2, $J = 17$ Hz, CH_2(2′)) and δ(C) 171.6 (O=C(1′)) suggested that **1** possesses a 3,4-dimethylpent-3-enoyl group in 11α-position. The presence of these substituents at C(11) and C(12) were further confirmed by means of ^{1}H,^{1}H-COSY and NOESY spectra. Thus, the structure of pachysanonin (**1**) was established as (3β,11α,12β)-12-acetoxy-3-(dimethylamino)-11-[(3,4-dimethylpent-3-enoyl)oxy]pregnan-20-one.

Pachysanone (**2**) showed the molecular ion at *m/z* 500.312909 (M^+), which was consistent with the formula $C_{30}H_{44}O_6$. The EI mass spectrum also exhibited $C_6H_{11}COO$ and AcO substituents, as deduced from the characteristic fragments at *m/z* 440 ($[M - AcOH]^+$), 373 ($[M - C_6H_{11}COO]^+$), and 313 ($[M - AcOH-C_6H_{11}COO]^+$). The IR spectrum showed absorptions for ketone and ester C=O groups at ν_{max} 1735 (br.), 1718, 1710, 1270, and 1240 cm^{-1}. The ^{1}H-NMR spectrum of **2** displayed the characteristic signals of a pregnanone, with resonances at δ(H) 0.950 (*s*, Me(18)), 1.116 (*s*, Me(19)), and 1.925 (*s*, Me(21)), 1.990 (*s*, Ac), 1.610 (*s*, Me) 1.639 (*s*, Me), 1.651(*s*, Me), 2.954/2.904 (A_2, $J = 16$ Hz, CH_2(2′)). In general, the ^{1}H-NMR spectrum of pachysanone (**2**) closely resembles that of pachysanonin (**1**), but lacks the Me_2N signal at δ(H) 2.210 (see *Table*).

From ^{13}C-NMR and DEPT experiments, compound **2** was found to give rise to 30 C-atoms, including seven Me, eight CH_2, and seven sp^3-hybridized CH groups, with two oxygenated CH resonances at δ(C) 72.1 and 82.3, as well as eight quaternary C-atoms, including two sp^2-hyridized C-atoms at 119.9 and 129.2, and four C=O resonances at

Table. *Diagnostic NMR Data of Compounds* **1** *and* **2**. In $CDCl_3$ at 500/125 MHz; δ in ppm, J in Hz. Primed atom numbers refer to the 3,5-pent-3-enoyl side chain (see chemical formulae).

Position/group	**1**		**2**	
	δ(C)	δ(H)	δ(C)	δ(H)
1	32.7 (*t*)		38.2 (*t*)	
2	24.9 (*t*)		38.0 (*t*)	
3	61.3 (*d*)		210.8 (*s*)	
4	31.8 (*t*)		44.8 (*t*)	
5	33.8 (*d*)		46.8 (*d*)	
6	28.9 (*t*)		29.2 (*t*)	
7	31.5 (*t*)		31.4 (*t*)	
8	33.8 (*d*)		33.9 (*d*)	
9	55.5 (*d*)		55.6 (*d*)	
10	37.9 (*s*)		37.3 (*s*)	
11	72.2 (*d*)	5.292 (*dd*, $J = 9.6$)	72.1 (*d*)	5.523 (*dd*, $J = 9.6$)
12	82.6 (*d*)	4.748 (*d*, $J = 9.6$)	82.3 (*d*)	4.783 (*d*, $J = 9.6$)
13	47.8 (*s*)		47.8 (*s*)	
14	52.8 (*d*)		52.6 (*d*)	
15	24.2 (*t*)		24.3 (*t*)	
16	26.2 (*t*)		26.2 (*t*)	
17	60.8 (*d*)	1.960 (*m*)	60.8 (*d*)	1.979 (*m*)
18	9.8 (*q*)	0.950 (*s*)	9.8 (*q*)	0.950 (*s*)
19	12.2 (*q*)	0.959 (*s*)	17.7 (*q*)	1.116 (*s*)
20	209.6 (*s*)		209.2 (*s*)	
21	30.8 (*q*)	1.930 (*s*)	30.9 (*q*)	1.925 (*s*)
Me_2N	43.6 (*q*)	2.210 (*s*)		
C(1')=O	171.6 (*s*)		171.4 (*s*)	
CH_2(2′)	40.5 (*t*)	2.936, 2.899 (A_2, $J = 17$)	40.5 (*t*)	2.954, 2.903 (A_2, $J = 16$)
C(3′)	120.3 (*s*)		119.9 (*s*)	
C(4′)	128.8 (*s*)		129.2 (*s*)	
Me(5′)	19.4 (*q*)	1.650 (*s*)	19.4 (*q*)	1.610 (*s*)
Me(6′)	20.6 (*q*)	1.669 (*s*)	20.6 (*q*)	1.639 (*s*)
Me(7′)	20.6 (*q*)	1.685 (*s*)	20.6 (*q*)	1.651 (*s*)
Me*C*O	171.2 (*s*)		171.2 (*s*)	
*Me*CO	21.0 (*q*)	2.007 (*s*)	21.0 (*q*)	1.990 (*s*)

δ(C) 210.8, 209.2, 171.4, and 171.2, respectively. The ^{13}C-NMR chemical shifts of rings *B*, *C*, and *D*, and the side-chain resonances of **2** also resemble those of **1**, except for the absence of both the 3-(Me_2N) and H−C(3) resonances (*Table*). The latter was replaced with a C=O resonance at δ(H) 210.8, suggesting a pregnan-3-one moiety, as confirmed by HMQC and HMBC techniques. From these data, the parent steroid structure of **2** was identified as 11α,12β-dihydroxypregnan-3,20-dione, which has been described in the literature [8]. The ^{1}H,^{1}H-COSY, NOESY, HMQC, and HMBC spectra of **2** indicated the same substitution patterns for the AcO and 3,4-dimethylpent-3-enoyl groups as for **1**. Accordingly, the structure of pachysanone (**2**) was determined as (11α,12β)-12-acetoxy-11-[(3,4-dimethylpent-3-enoyl)oxy]pregnane-3,20-dione.

Interestingly, **1** and **2** each bear a 3,4-dimethylpent-3-enoyl substituent at C(11), a functional group that is quite rare in natural products. Also, the biosynthetic pathway leading to 3,4-dimethylpent-3-enoic acid has not been discussed. To further confirm the structure of this substituent, we, thus, performed an X-ray crystallographic analysis of **2**

(*Figure*), which unequivocally corroborated the structure identified by spectroscopic methods.

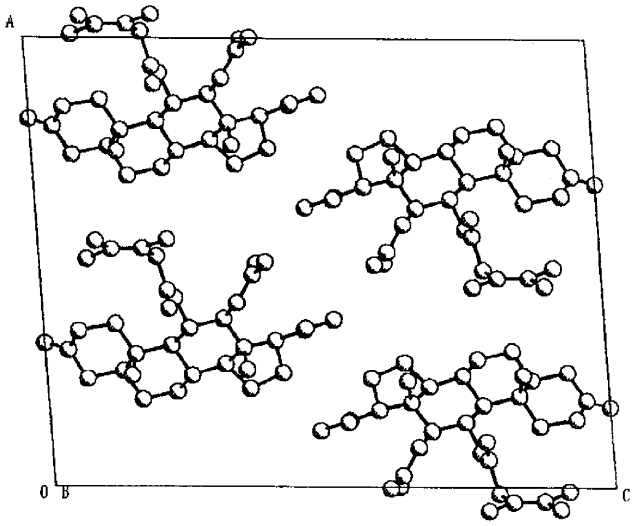


Figure. *X-Ray Single-Crystal Structure of Pachysanone* (**2**)

2.2. *Biology.* Compounds **1** and **2**, along with natural antitumor products such as harringtonine, homoharringtonine, and adriamycin (positive control) were evaluated for their biological effects on LLC (*Lewis* lung carcinoma) tumor cells. Pachysanone (**2**) and pachysanonin (**1**) showed significant activities against these tumor cells, with IC_{50} values of 0.020 ± 0.006 and 2.0 ± 0.3 μg/ml, respectively. The natural antitumor products harringtonine, homoharringtonine, and adriamycin showed IC_{50} values of 0.020 ± 0.007, 0.15 ± 0.04, and 0.06 ± 0.03 μg/ml, respectively, in this bioassay. The strong activity of pachysanone (**2**) against tumor cells is quite surprising, since not many similarly active steroidal compounds have been found so far. The observation that the alkaloidal analogue **1** is 100-fold less cytotoxic than **2** is most likely due to discrimination at the level of the macromolecular target.

Experimental Part

General. Column Chromatography (CC): Alumina (200–300 mesh; *Marine Chemical Industry Factory of Qingdao*, China), *Diaion HP-20* and *NH_2-Silica Gel* (*Mitsubishi Chemical Co.*, Tokyo). TLC: pre-coated *Silica Gel 60 F_{254}* plates (0.25 mm; *Merck*, Germany), visualization by spraying with 20% H_2SO_4 or *Dragendorff* reagent, followed by heating. M.p.: *XRC-1* Apparatus; uncorrected. IR Spectra: *JASCO FT/IR-230* spectrometer; in cm^{-1}. ^1H-, ^{13}C-, and 2D-NMR spectra: *Varian UNITY-500* spectrometer, at 500 MHz (^1H) and 125 MHz (^{13}C), in $CDCl_3$; chemical shifts δ in ppm rel. to Me_4Si as internal standard, coupling constants J in Hz. MS spectra: *JEOL D-300* and *VG Autospec 3000* spectrometers; in m/z (rel. %).

Plant Material. Pachyasandra axillaris FRENCH. was collected in Songming County, Yunnan Province, China, in 1985. A specimen (No. K198507287) was taxonomically identified by Prof. *Zhang Chang-Qi*, and deposited at the Herbarium of the Kunming Institute of Botany, the Chinese Academy of Sciences.

Extraction and Isolation. Air-dried whole plants (45 kg) of *P. axillaris* were cut into small pieces, and extracted with boiling 95% EtOH (3×). After concentration of the combined extracts, the residue was dissolved in 5% aq. AcOH, and the insoluble material was removed by filtration. The acidic soln. was basified

with 28% NH_4OH, and extracted thoroughly with $CHCl_3$. The org. layer was washed with H_2O, dried, and concentrated *in vacuo* to give a crude mixture of alkaloids (928 g). This material was dissolved in $CHCl_3$, and extracted with an equal volume of 3% aq. HCl soln. The org. layer was separated, washed with H_2O, dried, and concentrated *in vacuo* to give a weakly basic alkaloid fraction (560 g). The latter was extracted with acetone, and the insoluble material was separated by filtration. Then, the acetone soln. was concentrated *in vacuo*, and the residue was subjected repeatedly to CC (alumina; Et_2O/benzene 1:9, 2:8, and 3:7; then MeOH/$CHCl_3$ 1:9) to afford a fraction (500 mg) comprising 15 compounds. This fraction was re-subjected repeatedly to CC (NH_2–SiO_2; hexane/acetone 7:3) to give compounds **1** (150 mg) and **2** (30 mg).

Pachysanonin (=*(3β,11α,12β)-12-Acetoxy-3-(dimethylamino)-11-[(3,4-dimethylpent-3-enoyl)oxy]-pregnan-20-one*; **1**). Yield: 150 mg (3.3 ppm). Colorless needles (from acetone). M.p. 153.5–154.5° (dec.; acetone). UV: not active. IR (KBr): 3460, 3400 (sh), 2980, 2940, 2860, 2810, 2760, 2640, 1745, 1735, 1710, 1680, 1680, 1640, 1455, 1370, 1335, 1270, 1240, 1220, 1180, 1150, 1010. ^{1}H- and ^{13}C NMR ($CDCl_3$): see *Table*. EI-MS: 530 (90, $[M+H]^+$), 529 (15, M^+), 471 ($[M+1-AcOH]^+$), 403 (65), 402 (70), 342 (55), 297 (50), 255 (20), 145 (30), 110 (97), 84 (100), 71 (90), 58 (55), 56 (60). HR-EI-MS: 529.3786 (M^+, $C_{32}H_{51}NO_5^+$; calc. 529.3767).

Pachysanone (=*(11α,12β)-12-Acetoxy-11-[(3,4-dimethylpent-3-enoyl)oxy]pregnane-3,20-dione*; **2**). Yield: 30 mg (0.6 ppm). Colorless needles (from acetone). M.p. 122.5–124.5° (dec., acetone). UV: not active. IR (KBr): 3460, 3400, 2970, 2920, 2880, 1735, 1718, 1710, 1680, 1370, 1270, 1240, 1175, 1150, 1120, 1030. ^{1}H- and ^{13}C-NMR ($CDCl_3$): see *Table*. EI-MS: 500 (11, M^+), 440 (42, $[M-AcOH]$), 373(10), 313 (100), 295 (30), 270 (63), 203 (52), 161 (47), 145 (45), 110 (84), 95 (10), 83 (85), 67 (57). HR-EI-MS: 500.3129 (M^+, $C_{30}H_{44}O_6^+$; calc. 500.3138).

X-Ray Crystal-Structure Analysis of Pachysanone (**2**). Formula, $C_{30}H_{44}O_6$; M_r 500.31; crystal size 0.07 × 0.07 × 0.50 mm; monoclinic, space group *C*2, $a=18.998(7)$, $b=6.517(1)$, $c=23.475(6)$ Å, $\beta=94.68(1)°$; $V=2896.7(14)$ Å^3; $Z=4$, $D_c=1.148$ g/cm^3. The structural refinement was carried out by direct methods (SHELXS-86), and all the C- and O-atoms were positioned by the difference *Fourier* method, using full-matrix least squares. Refinement parameters: R_f (final) = 0.066, $Rw=0.062$, $S=3.739$ ($w=1/\sigma^2|F|$), GoF = 7.345 for 1324 obs. reflections ($|F|^2 \geq 8.0\sigma|F|^2$). $(\Delta/\sigma)_{max}=0.057$, $(\Delta\rho)_{min}=-0.180$ e/Å^3, $(\Delta\rho)_{max}=-0.180$ e/Å^3. The X-ray structure of **2** is shown in the *Figure*. In the crystal, rings *A*, *B*, and *C* are in chair conformations, with *trans* junctions between *A*/*B*, *B*/*C*, and *C*/*D*. CCDC-277110 contains the supplementary crystallography data for this paper. These data can be obtained free of charge *via* http://www.ccdc.cam.ac.uk/data_request/cif (or from the *Cambridge Crystallographic Data Centre*, 12 Union Road, Cambridge CB2 1EZ, UK; fax: +44 1223 336033; e-mail: deposit@ccdc.cam.ac.uk).

Cytotoxicity Assay. Lewis lung carcinoma (LCC) tumor cells were cultured in *RPMI-1640* medium containing 5% fetal bovine serum (FBS). For a sulforhodamine B (SRB) assay, the cells were cultured in *RPMI 1640* containing 7% of FBS. A cell suspension (100 µl; 40,000–50,000 cells/ml) in the culture medium was inoculated into each well of a 96-well microtiter plate. After 1 d, a time-zero control plate was made. Compounds were directly treated, and the cells were incubated for a further 48 h in a humidified 5% CO_2 atmosphere at 37°. The cells were fixed with 50% trichloroacetic acid (TCA; 50 µl) for 1 h at 4°, and the plates were washed with tap H_2O (5×), and air-dried. Then, SRB soln. (50 µl, 0.4% in 1% AcOH) was added, and staining was performed at r.t. for 30 min. The residual dye was washed out with 1% AcOH, and the plates were air-dried. To each well, 10 mM *Tris* buffer (100 µl, pH 10.5) was added. The optical density (OD) of each well was measured with a microtiter-plate reader at 540 nm [9]. The activities of compounds **1**, **2**, harringtone, homoharringtonine, and adriamycin (pos. control) were determined at 100, 10, 1, 0.1, and 0.01 mg/ml, resp. Growth inhibition was calculated as follows:

$\%\text{-Inhibition} = (OD_{compd} - OD_{blank})/(OD_{control} - OD_{blank}) \times 100$. The IC_{50} values (50% growth inhibition) were calculated by the *Probit* method [10].

M.-H. Q. is grateful to the *Peace Nakajima Foundation* for providing financial support as a foundation fellow. The authors also thank Prof. *Qitai Zheng* and *Yang Lu* of the Institute of Materia Medica, Chinese Academy of Medical Science, for the X-ray crystallographic analysis.

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Received January 18, 2005