Triterpenoid Alkaloids from *Buxus microphylla*

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Two new triterpenoid alkaloids, buxmicrophyllines J and K (**1** and **2**, resp.), together with four analogues, **3**–**6**, were isolated from the leaves and stems of *Buxus microphylla*. The structures of the new compounds were elucidated by NMR and MS spectroscopic analyses. The partial assignments of the NMR spectra of **3** were also revised. Compounds **1** and **3**–**6** were evaluated for their growth inhibitory activity against human cell lines HL-60, SMMC-7721, A-549, SK-BR-3, and PANC-1. Compound **6** showed significant cytotoxicity against HL-60, SK-BR-3, and PANC-1 cell lines, with IC_{50} values of 6.46, 19.61, and 28.57 μM, respectively.

Introduction. – *Buxus microphylla* SIEB. et ZUCC. (Buxaceae), is an evergreen shrub, widely distributed in the southern China. This plant is often used for the treatment of cardiovascular and cerebrovascular diseases, and against hypertension [1–3]. In previous studies, we found that the triterpenoid alkaloids from this plant showed cytotoxic activities [4]. Aimed at finding potential bioactive metabolites, we further explored this plant collected from Guodong County (Yunnan). As a result, two new triterpenoid alkaloids, buxmicrophyllines J and K (**1** and **2**, resp.), as well as four known ones, demethylcyclomikuranine (**3**) [5], buxbodine B (**4**) [6], cyclomicrobuxinine (**5**) [7], and *N*-acetyldihydrocyclomicrophylline F (**6**) [8] (*Fig. 1*) were obtained. In addition, compounds **1** and **3**–**6** were assayed for their cytotoxicity against human cell lines HL-60, SMMC-7721, A-549, SK-BR-3, and PANC-1. In this article, we report the isolation, structure elucidation, and cytotoxic activity of the triterpenoid alkaloids from *B. microphylla.*

Results and Discussion. – 1. *Structure Elucidation.* Compound **1** was obtained as a white powder, and the molecular formula was established as $C_{36}H_{54}N_2O_3$ on the basis of HR-ESI-MS at *m/z* 563.4175 ($[M+H]^+$; calc. 563.4212), with eleven degrees of unsaturation. The IR spectrum suggested the presence of NH (3473 cm^{-1}) and C=O (1715 cm^{-1}) functions.

The 1D-NMR spectrum (*Table 1*) of **1** showed resonances of three Me (δ(C) 18.9 (C(18)), 10.8 (C(29)), 19.6 (C(30))), eight CH_2 (δ(C) 32.3 (C(1)), 26.2 (C(2)), 19.8 (C(6)), 25.2 (C(7)), 27.5 (C(11)), 33.1 (C(12)), 44.5 (C(15)), 30.4 (C(19))), five CH (δ(C) 57.5 (C(3)), 45.0 (C(5)), 46.9 (C(8)), 80.4 (C(16)), 56.6 (C(17))), five quaternary C-atoms (δ(C) 37.4 (C(4)), 18.9 (C(9)), 25.9 (C(10)), 44.9 (C(13)), 47.8 (C(14))) in the

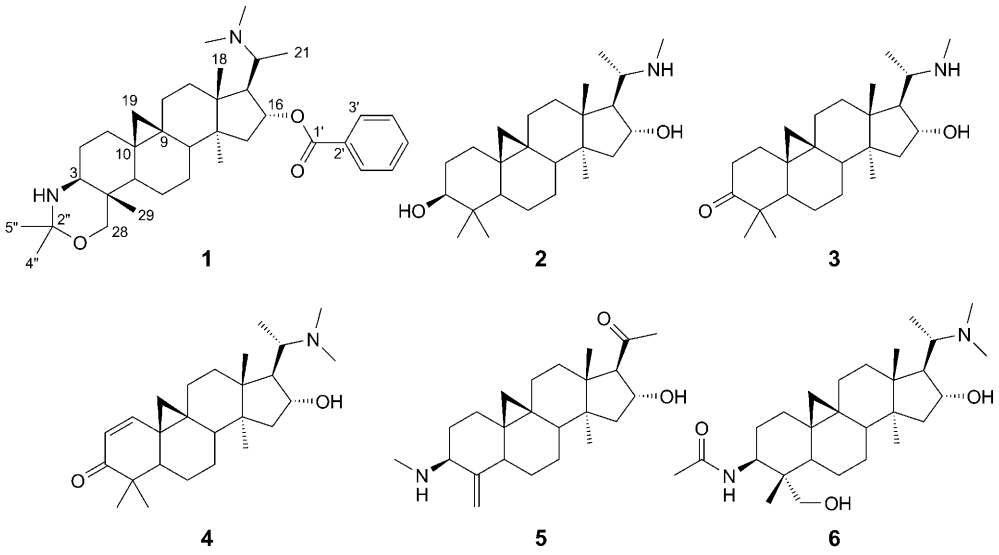

Fig. 1. *Chemical structures of compounds* **1**–**6**

Table 1. *^{1}H- and ^{13}C-NMR Data of* **1**. At 500/125 MHz, resp., in $CDCl_3$; *J* in Hz.

	δ(C)	δ(H)		δ(C)	δ(H)
CH_2(1)	32.3 (*t*)	1.32–1.37 (*m*), 1.80–1.82 (*m*)	CH_2(19)	30.4 (*t*)	0.35 (*d*, *J* = 4.0), 0.63 (*d*, *J* = 4.0)
CH_2(2)	26.2 (*t*)	1.30–1.34 (*m*), 1.47–1.50 (*m*)	H–C(20)	59.8 (*d*)	2.52–2.58 (*m*)
H–C(3)	57.5 (*d*)	2.78–2.82 (*m*)	Me(21)	9.6 (*q*)	0.84 (*d*, *J* = 6.0)
C(4)	37.4 (*s*)		CH_2(28)	71.5 (*t*)	3.50 (*s*)
H–C(5)	45.0 (*d*)	1.98–2.01 (*m*)	Me(29)	10.8 (*q*)	0.97 (*s*)
CH_2(6)	19.8 (*t*)	1.39–1.42 (*m*), 1.96–1.99 (*m*)	Me(30)	19.6 (*q*)	1.16 (*s*)
CH_2(7)	25.2 (*t*)	1.35–1.39 (*m*), 2.09–2.11 (*m*)	C(1′)	165.9 (*s*)	
H–C(8)	46.9 (*d*)	1.60–1.64 (*m*)	C(2′)	131.2 (*s*)	
C(9)	18.9 (*s*)		H–C(3′)	128.2 (*d*)	8.02 (*d*, *J* = 7.2)
C(10)	25.9 (*s*)		H–C(4′)	129.4 (*d*)	7.40–7.43 (*m*)
CH_2(11)	27.5 (*t*)	1.47–1.51 (*m*), 1.55–1.58 (*m*)	H–C(5′)	132.4 (*d*)	7.50–7.53 (*m*)
CH_2(12)	33.1 (*t*)	1.40–1.43 (*m*), 1.94–1.98 (*m*)	H–C(6′)	129.4 (*d*)	7.40–7.43 (*m*)
C(13)	44.9 (*s*)		H–C(7′)	128.2 (*d*)	8.02 (*d*, *J* = 7.2)
C(14)	47.8 (*s*)		C(2″)	84.1 (*s*)	
CH_2(15)	44.5 (*t*)	1.41 (*d*, *J* = 17.5)	Me(4″)	31.1 (*q*)	1.37 (*s*)
H–C(16)	80.4 (*d*)	5.32–5.35 (*m*)	Me(5″)	21.2 (*q*)	1.41 (*s*)
H–C(17)	56.6 (*d*)	2.30–2.33 (*m*)	Me_2N	40.4 (*q*)	2.08 (*s*)
Me(18)	18.9 (*q*)	1.02 (*s*)			

^{13}C-NMR spectrum, along with the characteristic cyclopropyl CH_2 H-atoms appearing as two *doublet*s at δ(H) 0.35 and 0.63 ($J(19\alpha,19\beta) = 4.0$ Hz), which confirmed the cycloartane-type triterpenoid skeleton as in the case of alkaloids isolated from the genus *Buxus* [9–11]. Moreover, six aromatic C-atoms (δ(C) 131.2, 128.2 × 2, 129.4 × 2, 132.4) and five aromatic H-atoms appeared at δ(H) 7.40–8.02. Together with the

HMBCs from H–C(3′/7′) (δ(H) 8.02) and H–C(4′/6′) (δ(H) 7.40–7.43) to the ester CO group C(1′) (δ(C) 165.9), this indicated the presence of a benzoyl moiety in **1** being positioned at C(16) on the basis of the linkage of H–C(16) (δ(H) 5.32–5.35) with C(1′) in the HMBC.

Considering the fact that five degrees of unsaturation have to be ascribed to the benzoyl group, another five ones were assigned to the cycloartane-type triterpenoid skeleton, and one more ring was required for the structure. This ring was proposed to be a dimethyl-substituted tetrahydroxazine by the HMBCs (*Fig. 2*) from CH_2(28) (δ(H) 3.50) to C(3), C(4), C(29), and C(2″) (δ(C) 84.1), from Me(4″) (δ(H) 1.37) to C(3), C(28), C(29), C(2″), and C(5″) (δ(C) 21.2), and from Me(5″) (δ(H) 1.41) to C(3), C(28), C(29), C(2″), and C(4″) (δ(C) 31.1). On the basis of the above evidence, constitutional formula of compound **1** was established.

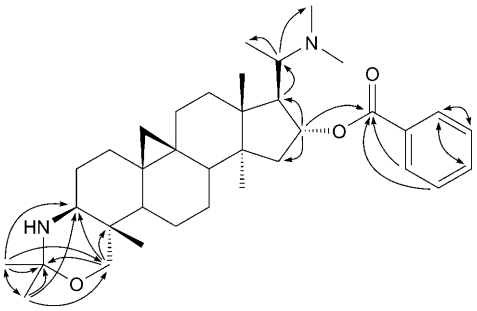

Fig. 2. *Key HMBCs* (H→C) *for compound* **1**

It has been reported that the H-atoms at C(5) and C(20) in *Buxus* alkaloids are α- and β-oriented, respectively [12–14]. Therefore, the configurations of H–C(28) and H–C(16) were accordingly determined as α and β, respectively, on the basis of the ROESY correlations between H–C(5) (δ(H) 1.98–2.01) and H–C(28), and between H–C(20) (δ(H) 2.52–2.58) and H–C(16). Thus, the structure of buxmicrophylline J was deduced as **1**.

Compound **2** was crystallized from acetone as colorless needles. The molecular formula was determined as $C_{25}H_{43}NO_2$ by positive-mode HR-ESI-MS (*m*/*z* 390.3369 ($[M+H]^+$; calc. 390.3372)) and NMR spectra. The ^{13}C-NMR and DEPT spectra of **2** showed resonances for 25 C-atoms including six Me, eight CH_2, six CH, and five quaternary C-atoms (*Table 2*), which were similar to those of cyclovirobuxine A [15] except that the Me_2N group at C(20) in cyclovirobuxine A was replaced by a NHMe group in **2**. The observation of NOESY correlations of Me(28) (δ(H) 0.87), H–C(3) (δ(H) 3.20–3.24), and H–C(5) (δ(H) 1.38–1.42), established the α-orientation of the H-atom at C(3). Hence, the structure of buxmicrophylline K was established as **2**.

Compound **3** was obtained as colorless needles. The molecular formula was established to be $C_{25}H_{41}NO_2$ on the basis of ESI-MS ($[M+H]^+$ *m*/*z* 388) and NMR spectra (*Table 2*), which was consistent with the molecular formula in the literature [5]. The present data of H–C(16) (δ(H) 4.25–4.29, δ(C) 75.4) fit the proposed structure better than those reported (δ(H) 1.44, 1.19, δ(C) 30.4). So the data of **3** were revised as the structure of demethylcyclomikuranine.

Table 2. *^{1}H- and ^{13}C-NMR Data of* **2** *and* **3**. At 500/125 MHz, resp., in $CDCl_3$; δ in ppm, J in Hz.

	2		**3**	
	δ(C)	δ(H)	δ(C)	δ(H)
CH_2(1)	31.8 (*t*)	1.49–1.55 (*m*), 1.67–1.70 (*m*)	33.0 (*t*)	1.40–1.43 (*m*), 1.73–1.77 (*m*)
CH_2(2)	26.0 (*t*)	2.01–2.04 (*m*)	37.1 (*t*)	2.03–2.07 (*m*), 2.61–2.65 (*m*)
H–C(3) or C(3)	78.3 (*d*)	3.20–3.24 (*m*)	217.4 (*s*)	
C(4)	40.3 (*s*)		50.0 (*s*)	
H–C(5)	46.9 (*d*)	1.38–1.42 (*m*)	47.4 (*d*)	1.42–1.44 (*m*)
CH_2(6)	20.8 (*t*)	1.56–1.59 (*m*)	21.1 (*t*)	0.72–0.76 (*m*), 1.35–1.38 (*m*)
CH_2(7)	25.8 (*t*)	1.23–1.27 (*m*)	25.7 (*t*)	1.01–1.04 (*m*)
H–C(8)	47.6 (*d*)	1.21–1.24 (*m*)	48.1 (*d*)	1.61–1.65 (*m*)
C(9)	18.8 (*s*)		20.0 (*s*)	
C(10)	26.2 (*s*)		25.9 (*s*)	
CH_2(11)	30.0 (*t*)	1.18–1.22 (*m*)	26.0 (*t*)	1.47–1.51 (*m*)
CH_2(12)	31.7 (*t*)	1.13–1.16 (*m*)	31.4 (*t*)	1.48–1.52 (*m*), 1.67–1.70 (*m*)
C(13)	45.8 (*s*)		45.7 (*s*)	
C(14)	47.4 (*s*)		47.2 (*s*)	
CH_2(15)	46.8 (*t*)	1.90–1.94 (*m*)	46.7 (*t*)	1.36–1.41 (*m*), 1.88–1.91 (*m*)
H–C(16)	75.8 (*d*)	4.27–4.30 (*m*)	75.4 (*d*)	4.25–4.29 (*m*)
H–C(17)	56.4 (*d*)	2.07–2.09 (*m*)	56.2 (*d*)	2.00–2.04 (*m*)
Me(18)	18.9 (*q*)	0.95 (*s*)	20.5 (*q*)	0.94 (*s*)
CH_2(19)	29.7 (*t*)	0.28 (*d*, $J = 4.0$), 0.48 (*d*, $J = 4.0$)	29.6 (*t*)	0.51 (*d*, $J = 4.0$), 0.69 (*d*, $J = 4.0$)
H–C(20)	58.9 (*d*)	3.28–3.33 (*m*)	58.8 (*d*)	3.26–3.29 (*m*)
Me(21)	14.6 (*q*)	1.24 (*d*, $J = 6.4$)	14.5 (*q*)	1.25 (*d*, $J = 6.4$)
Me(28)	25.1 (*q*)	0.87 (*s*)	21.8 (*q*)	0.99 (*s*)
Me(29)	13.8 (*q*)	0.71 (*s*)	18.9 (*q*)	0.94 (*s*)
Me(30)	20.3 (*q*)	1.06 (*s*)	20.1 (*q*)	1.05 (*s*)
MeN	29.0 (*q*)	2.59 (*s*)	29.0 (*q*)	2.60 (*s*)

2. *Cytotoxicity.* Compounds **1** and **3**–**6** were tested for their growth inhibitory ability against human cell lines HL-60, SMMC-7721, A-549, SK-BR-3, and PANC-1. Compared with positive control DDP, both **3** and **6** were found to be active against SK-BR-3 cell lines (*Table 3*). Compound **6** also showed moderate activity against the cell lines HL-60 and PANC-1.

Table 3. *Cytotoxicity of Compounds* **1** *and* **3**–**6**, *given as* IC_{50} *Values* [μM]

	Cell lines				
	HL-60	SMMC-7721	A-549	SK-BR-3	PANC-1
1	>40	>40	>40	>40	>40
3	>40	>40	>40	39.06	>40
4	>40	>40	>40	>40	>40
5	>40	>40	>40	>40	>40
6	6.46	>40	>40	19.61	28.57
DDP	1.67	19.36	29.70	17.38	37.97

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Experimental Part

General. Petroleum ether (PE) for chromatography had a b.p. range of 60–90°. Column chromatography (CC): silica gel (SiO_2, 200–300 mesh; *Qingdao Marine Chemical, Inc.*), amino SiO_2 (75–100 μm, *Fuji Silysia Chemical Ltd.*, Japan); *Lichroprep RP-18* (40–63 μM; *Merck*), or *Sephadex LH-20* (*Pharmacia*). M.p.: *Yu-Hua X-4* melting point apparatus. Optical rotations: *Horiba SEAP-300* spectropolarimeter. IR Spectra: *Shimadzu IR-450* instrument, KBr pellets. NMR Spectra: *Bruker AV-400* and *DRX-500* instruments. HR-ESI-MS: *VG Autospec-3000* spectrometer; in *m/z*.

Plant Material. The stems and leaves of *B. microphylla* were collected at Guodong County (Yunnan), P. R. China, in September 2007, and identified by Prof. *Xi-Wen Li* of the Kunming Institute of Botany. A voucher specimen (KIB. 20070915) was deposited with the State Key Laboratory of Phytochemistry and Plant Resources in West China, Kunming Institute of Botany, Kunming, P. R. China.

Extraction and Isolation. The dried and powdered plant material of *B. microphylla* (5.0 kg) was extracted with MeOH (3 × 10 l) at reflux, and the extract was adjusted to pH 2 with 2‰ HCl (4 l). The acidic mixture was defatted with AcOEt (3 × 2 l). The aq. layer was alkalinized to pH 10 with 5% NaOH (200 ml) followed by exhaustive extraction with $CHCl_3$ (4 × 2.5 l) to obtain a $CHCl_3$-soluble fraction (45 g). CC (SiO_2; $CHCl_3$/MeOH 1:0 → 1:1) afforded four fractions: *Frs. 1–4*. *Fr. 2* (11.8 g) was subjected to CC (SiO_2; PE/AcOEt 20:1 → 5:1) to provide three subfractions: *Frs. 2.1–2.3*. *Fr. 2.1* (2 g) was further separated by *Sephadex LH-20* eluted with MeOH and CC (amino SiO_2; PE/AcOEt 10:1) to afford **3** (25 mg). *Fr. 2.2* (1.2 g) was further separated on RP_{18} CC with aq. MeOH (60–90%) to yield **4** (43 mg). *Fr. 3* (14 g) was chromatographed over CC (SiO_2; $CHCl_3$/MeOH 20:1 → 5:1): four subfractions. *Fr. 3.1* (2 g) was further repeatedly separated on CC (amino SiO_2; $CHCl_3$/MeOH 30:1) to give **1** (11 mg). *Fr. 3.2* (3 g) was further separated by *Sephadex LH-20* eluted with MeOH to give *Frs. 3.2.1* and *3.2.2*. *Fr. 3.2.1* was recrystallized from MeOH: **6** (16 mg). *Fr. 3.2.2* was purified by CC (SiO_2; $CHCl_3$/MeOH 10:1) to give **2** (4 mg) and **5** (20 mg).

Buxmicrophylline J (=*(4a*R*,6a*S*,7a*R*,9a*S*,10*R*,11*S*,12a*R*,14b*R*)-10-[1-(Dimethylamino)-ethyl]hexadecahydro-3,3,9a,12a,14b-pentamethyl-1*H*,3*H*-cyclopenta[7,8]cyclopropa[4a,4b]phenanthro-[2,1-*d*][1,3]oxazin-11-yl Benzoate*; **1**). White powder. M.p. 237°. $[\alpha]_D^{24} = 13.0$ ($c = 1.08$, $CHCl_3$). UV (MeOH): 203 (2.97), 272 (1.75). IR (KBr): 3473, 3266, 1715, 1632. ^{1}H- and ^{13}C-NMR: see *Table 1*. ESI-MS: 563 ($[M+H]^+$). HR-ESI-MS: 563.4175 ($[M+H]^+$, $C_{36}H_{55}N_2O_3^+$; calc. 563.4212).

Buxmicrophylline K (=*(3β,9β,16α,20*R*)-4,4,14-Trimethyl-20-(methylamino)-9,19-cyclopregnane-3,16-diol*; **2**). Colorless needles. M.p. 259°. ^{1}H- and ^{13}C-NMR: see *Table 2*. ESI-MS: 390 ($[M+H]^+$). HR-ESI-MS: 390.3369 ($[M+H]^+$, $C_{25}H_{44}NO_2^+$; calc. 390.3372).

Demethylcyclomikuranine (=*(9β,16α,20*R*)-16-Hydroxy-4,4,14-trimethyl-20-(methylamino)-9,19-cyclopregnan-3-one*; **3**). Colorless needles. ^{1}H- and ^{13}C-NMR: see *Table 2*. ESI-MS: 388 ($[M+H]^+$).

Cell Culture and Cytotoxicity Assay. A panel of human tumor cell lines was used: promyelocytic leukemia HL-60, hepatocellular carcinoma SMMC7721, alveolar basal epithelial carcinoma A549, breast carcinoma SK-BR-3, and pancreatic carcinoma PANC-1. The cell lines were obtained from Shanghai cell bank of China. All the cells were cultured in *RPMI-1640* or *DMEM* medium (*Hyclone*, USA), supplemented with 10% fetal bovine serum (*Hyclone*, USA) at 37° 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 with the MTT (=3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; *Sigma*, USA) method described as before [16], and with cisplatin (=*cis*-dichlorodiammine-platinum, DDP; *Sigma*, USA) as control. Cell growth inhibition curve was graphed and IC_{50} value of each compound was calculated by the *Reed* and *Muench* method [17].

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