Triterpenoid alkaloid derivatives from *Buxus rugulosa*

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Abstract: Four new triterpenoid alkaloid derivatives, buxrugulines A–D (1–4), together with four known ones (5–8), were isolated from the leaves and stems of *Buxus rugulosa*. The structures of compounds 1–4 were elucidated by NMR and MS spectroscopic analysis. All compounds were assayed for their cytotoxities against HL-60, SMMC-7721, A549, MCF-7, and SW480 cells lines.

Keywords: *Buxus rugulosa*, triterpenoid alkaloid derivatives, buxruguline, cytotoxicity

Introduction

*Buxus rugulosa*, belonging to the *Buxus* genus of the family Buxaceae, is a dwarf shrub growing in the rocky mountains in the northwest district of Yunnan Province. In previous phytochemical investigations of the genus *Buxus*, more than 220 triterpenoid alkaloid derivatives have been isolated. This type of alkaloid showed interesting pharmacological activities such as anti-myocardial ischemia, antibacterial activities, and inhibition of cholinesterases. In our previous studies from *Buxus* plants, new alkaloids with diverse structures and promising cytotoxic activities have been reported. As part of this study, we have examined the stems and leaves of *B. rugulosa*, and consequently isolated four new triterpenoid alkaloid derivatives, buxrugulines A–D (1–4), along with four known ones, *N*-acetoxy-cyclovirobuxin D (5), (+)-16α-acetoxybuxabenzamidienine (6), moenjodaramine (7), and irehine (8). Herein we report the isolation and structural elucidation of the new compounds, as well as cytotoxic activities of the isolates from *B. rugulosa*.

Results and Discussion

A crude alkaloid fraction of *B. rugulosa* yielded eight triterpenoid alkaloid derivatives by repeated silica gel, amino silica gel, C-18 and Sephadex LH-20 chromatography.

Buxruguline A (1) was obtained as white powder. Its molecular formula, C_{21}H_{26}NO, was established on the basis of HRESIMS analysis ([M + H]+, m/z 368.2944). The 1H NMR (Table 1) spectrum featured one N-methyl singlet at δ_H 2.47, four singlets at δ_H 0.80, 1.05, 1.17, and 1.26, corresponded to four tertiary methyl groups, and one doublet at δ_H 0.76 (6.5, H-21). The 13C NMR spectrum exhibited 25 carbon signals containing six quaternary carbons (one carbonyl carbon at δ_C 206.8 and two olefinic carbons at 146.1 and 146.3), eight methines (four olefinic carbons at δ_C 127.6, 127.7, 128.4, and...
129.7), five methylenes, and six methyl groups. Comparison of the spectroscopic data of 1 and cyclobutoxtriene revealed similarities except for the absence of a methyl on the nitrogen at C-20 and the presence of a double bond at C-6/7 in 1. This was supported by the HMBC correlations of H-5 (δH 2.73) with C-6 (δC 128.4) and of H-8 (δH 1.91) with C-7 (δC 127.6), C-9 (δC 146.1) (Figure 2). Therefore, 1 was elucidated as shown, and named buxruguline A.

**Figure 2.** Key HMBC correlations for compound 1.

Buxruguline B (2) was obtained as colorless needles. The HRESIMS exhibited a quasi-molecular ion peak at m/z 370.3105 ([M + H]+, calc. 370.3109), indicating the molecular formula C25H33NO. The 1H NMR spectrum featured three singlets for the three tertiary methyl groups at δH 0.85, 0.90, and 1.08, and the characteristic cyclopropylmethylene protons appeared as two doublets at δH 0.64 and 0.50 (4.1). The 1H and 13C NMR spectrum of 2 displayed the presence of a terminal methylene [δH 4.61, 4.85 (each, 1H, s); δC 153.3 (C), 101.5 (CH3)]. All the data indicated that compound 2 was similar to buxpinine, and the distinct difference between them was that an oxygenated methine (δH ≈ 72) of C-16 in buxpinine was replaced by a methylene (δH 34.2) in 2. This deduction was supported by HMBC correlations from H-21 (δH 1.08) to C-20 (δC 212.9, C-16 (δC 34.2) and from H-17 (δH 2.66) to C-20, C-16 and C-13 (δH 42.7). H-5 is invariably α-oriented in this type alkaida, the ROESY correlation of H-3 (δH 2.85) with H-5 (δH 2.12) indicating an α-orientation of H-3 and β-orientation of the amino functionality. So, the structure of 2 was elucidated as shown in Figure 1.

Buxruguline C (3) had the molecular formula C29H43NO3, as determined by HRESIMS analysis ([M + H]+, m/z 461.3749). The 1H NMR spectrum of 3 showed the presence of three methyl singlets at δH 0.98, 0.60, and 0.92 for the H-18, H-31, and H-32, a doublet methyl at δH 1.01 (6.0, 21-CH3), two N-methyl singlets at δH 2.45 and 2.88, together with characteristic protons due to one hydroxymethylene (δH 2.93 and 3.25), and one oxygenated methine (δH 4.27). These spectral data were quite similar to those of dihydroxycolobaleubuxine, except for the resonance of acetyl group [δH 1.92; δC 172.2 (C) and 22.3 (CH3)] attributable to the nitrogen at C-3. The relative configuration of 3 was elucidated by the ROESY experiment and comparison with other naturally occurring triterpenoid alkaloids possessing β-configuration of the amino group at C-3, H-5α, and H-20β. The ROESY correlations of H-5 (δH 1.84), and H-3 (δH 2.98) with H-30 (δH 2.93 and 3.25), and of H-20 (δH 2.97) with H-16 (δH 4.27) indicated that H-30 was in α-orientation, while H-16 was in β-orientation, respectively. Thus, the structure of buxruguline C was established as 3.

The molecular formula of buxruguline D (4) was assigned as C30H42N2O3 on the basis of the NMR data (Table 1) and HRESIMS. Comparison of the spectroscopic data of 4 and 3 revealed similarities cycloartane-type triterpenoid skeleton. The notable difference was that a OH functionality at C-16 in 3 was replaced by acetoxy group in 4, which confirmed by the downfielded H-16 (δH 4.11) proton signal and the HMBC correlation from H-16 to the O-acetyl carbonyl carbon at δC 169.3 (C). Moreover 4 has one less hydroxyl function at C-30 and one less methyl group on the nitrogen at C-3 than 3. Consequently, compound 4 was elucidated as shown and has been accorded the trivial name buxruguline D.

Biologically, all compounds were tested for their cytotoxicity against the HL-60, SMMC-7721, A549, MCF-7, and SW480 cells lines (Table 2). Compounds 6, 7 and 8 showed the better cytotoxic potential against A-549, and SW480 cell lines. Compounds 1–4 were noncytotoxic, with IC50 values > 40 μmol for all tested cell lines.

**Experimental Section**

**General Experimental Procedures.** Melting points were determined on a YU-HUA X-4 melting point apparatus. Optical rotations were obtained with a Horiba SEAP-300 polarimeter. Infrared spectra were recorded on a Shimadzu IR-450 instrument by using KBr pellets. NMR spectra were measured on a Bruker AV-400 and DRX-500 instrument (Bruker, Zürich, Switzerland) with TMS as internal standard. HR-ESIMS data were recorded on a VG Auto Spec-3000 spectrometer. Silica gel (200–300 mesh, Qingdao Marine Chemical, Inc), amino silica gel (75–100 μm, Fuji Silysia Chemical LTD, Japan), C-18 (20–45 μm, Fuji Silysia Chemical, LTD, Japan), and Sephadex LH-20 (Pharmacia) were used for column chromatography.

**Plant Material.** Buxus rugulosa were collected at Lijiang (Yunnan), China, in February 2008. The sample was identified by Prof. Xi-Wen Li of the Kunming Institute of Botany, and a voucher specimen (KIB 20080210) has been deposited at the State Key Laboratory of Phytochemistry and Plant Resources in West China, Kunming Institute of Botany, Chinese Academy of Sciences.

**Extraction and Isolation.** The materials of *B. rugulosa* (75.0 kg) were extracted with 90% MeOH under reflux, the combined extracts were partitioned between EtOAc and 0.001 mol/L HCl (pH ≈ 3.0). The aqueous layer was alkalized to pH 10.0 with 2 mol/L NaOH followed by exhaustively extraction with CHCl3. The CHCl3-soluble fraction (180 g) was chromatographed on a silica gel column, eluted with CHCl3-MeOH (1:0-1:1), to give four fractions (FA–FD). FB (45 g) was chromatographed on silica gel using petroleum ether (PE)-EtOAc (8:1) as solvent and repeated Sephadex LH-20 eluted with MeOH to yield 7 (19 mg). After column chromatography on C-18 gel column chromatography with aqueous MeOH (60%-90%), amino silica gel column with PE-EtOAc (10:1) and CHCl3-MeOH (50:1), further separated by Sephadex LH-20 eluted with MeOH, FC (14 g) to afford 1 (11 mg), 4 (4 mg), 5 (6 mg), 7 (28 mg), 8 (42 mg). FD (12 g) was chromatographed on silica gel using CHCl3-MeOH (10:1, 5:1) as gradi-
ent, and was further repeatedly separated on amino silica gel column chromatography, eluted with CHCl₃-MeOH (20:1, 10:1), to give 2 (6 mg), 3 (12 mg), 6 (76 mg).

**Buxrangline A (1):** white powder; mp 188–190°C; [α]₁₅° + 15.6 (c 1.04, CHCl₃); UV (MeOH) _λ_{max} (log ε) 208 (3.78), 244 (2.21) nm; IR (KBr) _ν_{max}: 1734 cm⁻¹; _¹H, _¹³C NMR data see Table 1; EIMS _m/z: 367, HRESIMS _m/z: 368.2944 [M + H]⁺ (calsed for C₂₅H₂₃NO [M + H]⁺, 368.2953).

**Buxrangline B (2):** colorless needle; mp 222–224 °C; [α]₁₅° + 18.4 (c 1.21, CHCl₃); UV (MeOH) _λ_{max} (log ε) 205 (3.59) nm; IR (KBr) _ν_{max}: 1735 cm⁻¹; _¹H, _¹³C NMR data see Table 1; ESIMS _m/z: 370 [M + H]⁺; HRESIMS _m/z: 370.3105 (calsed for C₂₅H₂₃NO [M + H]⁺, 370.3109).

| position | _δ_¹H, type | _δ_¹H | _δ_¹³C, type | _δ_¹³C | _δ_¹H, type | _δ_¹H | _δ_¹³C, type | _δ_¹³C |
|----------|-------------|-------|---------------|--------|-------------|-------|---------------|--------|
| 1a       | 29.7, CH₃   | 2.04, overlap | 31.0, CH₃   | 1.82, overlap | 32.4, CH₂ | 1.56, overlap | 31.6, CH₁ | 1.65, overlap |
| 1b       | 46.6, CH₂   | 2.34, m     | 25.7, CH₂   | 1.73, overlap | 27.0, CH₂ | 1.55, overlap | 25.6, CH₁ | 1.33, overlap |
| 2a       | 41.1, CH    | 2.73, d (3.5) | 41.9, CH   | 2.12, m     | 40.2, CH₂ | 1.84, s      | 50.5, CH₁ | 2.16, s      |
| 2b       | 128.4, CH₂  | 5.62, m     | 21.2, CH₂   | 1.80, overlap | 20.5, CH₂ | 1.62, overlap | 20.9, CH₁ | 1.50, overlap |
| 3        | 127.6, CH   | 5.42, m     | 26.2, CH₂   | 1.58, overlap | 25.4, CH₂ | 1.22, overlap | 28.4, CH₁ | 1.75, m      |
| 4        | 146.1, C    | 23.3, C     | 19.0, C     | 1.35, overlap | 47.3, CH | 1.44, overlap |
| 5        | 146.3, C    | 32.0, C     | 25.5, C     | 1.12, overlap | 1.12, overlap | 1.40, overlap | 28.4, CH₁ | 1.75, m      |
| 6        | 127.7, CH   | 5.69, m     | 21.4, CH₂   | 1.50, overlap | 25.8, CH₂ | 2.04, m      | 26.1, CH₁ | 1.33, overlap |
| 7        | 30.2, CH₂   | 1.60, overlap | 33.3, CH₂   | 1.72, overlap | 31.7, CH₂ | 1.62, overlap | 32.2, CH₁ | 1.22, overlap |
| 8        | 39.5, C     | 42.7, C     | 45.6, C     | 1.55, overlap | 1.44, overlap | 1.33, overlap | 49.9, C   |
| 9        | 41.5, C     | 46.0, C     | 47.7, C     | 1.55, overlap | 1.44, overlap | 1.33, overlap | 48.0, C   |
| 10       | 24.8, CH₂   | 2.04, overlap | 22.4, CH₂   | 1.52, overlap | 25.5, CH₂ | 1.82, overlap | 45.0, CH₁ | 1.38, overlap |
| 11       | 26.0, CH₂   | 1.32, overlap | 34.2, CH₂   | 2.12, m     | 75.8, CH | 4.27, m      | 78.5, CH | 4.11, m      |
| 12       | 47.5, CH    | 1.88, overlap | 46.8, CH    | 2.66, m     | 56.1, CH | 1.93, m      | 57.0, CH | 1.88, m      |
| 13       | 19.7, CH₁   | 0.80, s     | 18.3, CH₂   | 0.85, s     | 18.9, CH₁ | 1.08, s      | 19.0, CH₁ | 0.97, s      |
| 14       | 129.7, CH   | 6.51, s     | 22.0, CH₂   | 0.50, d (4.1) | 0.48, d (4.1) | 0.49, d (4.1) | 0.10, d (4.1) | 0.11, d (4.1) |
| 15       | 68.1, CH    | 2.09, m     | 212.9, C    | 64.1, CH | 2.97, overlap | 62.7, CH | 2.71, m      |
| 16       | 15.3, CH₃   | 0.76, d (6.5) | 17.8, CH₁   | 1.08, s     | 10.3, CH₁ | 1.01, d (6.0) | 9.8, CH₁ | 0.91, d (6.5) |
| 17       | 30.2, CH₁   | 1.26 (s)    | 101.5, CH₂  | 4.85, s     | 63.7, CH | 3.25, m      | 11.4, CH₁ | 0.76, s      |
| 18       | 35.3, CH₁   | 2.47, s     | 30.5, CH₂   | 2.93, m     | 2.94, m     | 2.95, m     | 2.96, m     | 2.97, m      |

[^H and ^¹³C NMR spectra were acquired at 500 (CDCl₃) and 125 MHz (CDCl₃), respectively.]
Table 2. Cytotoxicity data of compounds 1–8 with IC_{50} values (μM).

| No. | HL-60 | SMMC-7721 | A-549 | MCF-7 | SW480 |
|-----|-------|-----------|-------|-------|-------|
| 1   | > 40  | > 40      | > 40  | > 40  | > 40  |
| 2   | > 40  | > 40      | > 40  | > 40  | > 40  |
| 3   | > 40  | > 40      | > 40  | > 40  | > 40  |
| 4   | > 40  | > 40      | > 40  | > 40  | > 40  |
| 5   | 27.18 | > 40      | > 40  | > 40  | > 40  |
| 6   | 15.23 | 28.99     | 19.39 | 14.39 | 14.69 |
| 7   | 17.52 | > 40      | 19.70 | > 40  | 14.25 |
| 8   | 21.35 | > 40      | 23.52 | > 40  | 17.18 |
| cisplatin | 1.00 | 17.05     | 26.75 | 14.97 | 16.88 |

Buxruguline C (3): colorless needle; [α]_{D}^{24} + 8.8 (c 0.89, CHCl_{3}); UV (MeOH) λ_{max} (log ε) 205 (3.53) nm; IR (KBr) ν_{max} = 1698 cm^{-1}; H, 13C NMR data see Table 1; ESI-MS m/z 461 [M + H]^+; HRESIMS m/z 461.3749 (calcd for C_{22}H_{28}N_{2}O_{3}, [M + H]^+, 461.3743).

Buxruguline D (4): white powder; [α]_{D}^{24} + 17.5 (c 0.72, CHCl_{3}); UV (MeOH) λ_{max} (log ε) 203 (3.67) nm; IR (KBr) ν_{max} = 1696 cm^{-1}; H, 13C NMR data see Table 1; EIMS m/z 472 [M + H]^+; HRESIMS m/z 473.7114 (calcd for C_{22}H_{28}N_{2}O_{3} [M + H]^+, 473.7109).

Cell Culture and Cytotoxicity Assay. A panel of human tumor cell lines was used: promyelocytic leukemia HL-60, hepatocellular carcinoma SMMC-7721, alveolar basal epithelial carcinoma A549, breast adenocarcinoma MCF-7, and colon cancer SW480. The cells lines were obtained from the 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°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 the living cells with the MTT (MTT, sigma, USA) method described before, and using cisplatin (DDP, sigma, USA) as a positive control. Growth inhibition curve was graphed and the IC_{50} value of each compound was calculated by the Reed and Muench method.

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References

[1] Yan, Y. X.; Sun, Y.; Li, Z. R.; Zhou, L.; Qiu, M. H. Curr. Bio. Comps. 2011, 7, 47–64.
[2] Krishna, P. D.; Bruno, N. L.; Patrice, A. F.; Norbert, S. Nat. Prod. Rep. 2008, 25, 612–630.
[3] Hu, D.; Liu, X. Y.; Wang, Y. Y.; Chen, S. Z. Eur. J. Pharmacol. 2007, 569, 103–105.
[4] Chen, Q. W.; Shan, H. L.; Wang, H.; Li, Z.; Yang, B. F. J. Chin. Pharm. Sci. 2003, 12, 142–147.
[5] Atta-ur-Rahman; Choudhary, M. I.; Naz, S.; Ata, A.; Sener, B.; Turkoz, S. J. Nat. Prod. 1997, 60, 770–774.
[6] Atta, A.; Naz, S.; Choudhary, M. I. Atta-ur-Rahman; Sener, B.; Turkoz, S. Z. Naturforsch. 2002, 57c, 21–28.
[7] Choudhary, M. I.; Shahnaz, S.; Parveen, S.; Khalid, A.; Mesea, M. A.; Ayatollahi, S. A. M.; Atta-ur-Rahman. Chem. Biodiversity 2006, 3, 1039–1052.
[8] Atta-ur-Rahman; Parveen, S.; Khalid, A.; Parveen, M. I. Phytochemistry 2001, 58, 963–968.
[9] Atta-ur-Rahman; Parveen, S.; Khalid, A.; Farooq, A.; Ayatollahi, S. A. M.; Choudhary, M. I. Heterocycles 1998, 49, 481–488.
[10] Yan, Y. X.; Chen, J. C.; Sun, Y.; Wang, Y. Y.; Su, J.; Li, Y.; Qiu, M. H. Chem. Biodiversity 2010, 7, 1822–1827.
[11] Yan, Y. X.; Hu, X. D.; Chen, J. C.; Sun, Y.; Zhang, X. M.; Qing, C.; Qiu, M. J. Nat. Prod. 2009, 72, 308–311.
[12] Deng, L.; Huang, H.; Xu, M. X.; Zhou, S. Q.; Wang, X. W.; Lu, M.; Ren, F.; Li, D. Q. Acta Pharm. Sin. 2004, 39, 434–438.
[13] Choudhary, M. I.; Atta-ur-Rahman; Freyer, A. J.; Shamma, M. Tetrahedron 1986, 42, 5747–5752.
[14] Atta-ur-Rahman; Noor-e-ain, F.; Parveen, Z.; Türköz, S.; Sener, B. J. Nat. Prod. 1997, 60, 976–981.
[15] Atta-ur-Rahman; Nisa, M.; Farhi, S. Plant Med. 1983, 49, 126.
[16] Atta-ur-Rahman; Nisa, M.; Farhi, S. Naturforsch. 1984, 39b, 524–527.
[17] Babar, Z. U.; Ata, A.; Meshkatalsadat, M. H. Steroids 2006, 71, 1045–1051.
[18] Sangare, M.; Khuong-Huu, F.; Herlem, D.; Milliet, A.; Septe, B.; Berenger, G.; Lukacs, G. Tetrahedron Lett. 1975, 22/23, 1791–1794.
[19] Brown, K. S.; Khuong-Huu, F.; Hellmer, D.; Milliet, A.; Septe, B.; Berenger, G.; Lukacs, G. Tetrahedron Lett. 1965, 65, 71–74.
[20] Alley, M. C.; Scudiero, D. A.; Monks, A.; Okun, M. L.; Czerwinski, M. J.; Fine, D. L.; Abbott, B. J.; Mayo, J. G.; Shoemaker, R. H.; Boyd, M. R. Cancer Res. 1988, 48, 589–601.
[21] Reed, L. J.; Muench, H. Am. J. Hyg. 1938, 27, 493–497.