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Kalanchosides A–C, New Cytotoxic Bufadienolides from the Aerial Parts of *Kalanchoe gracilis*

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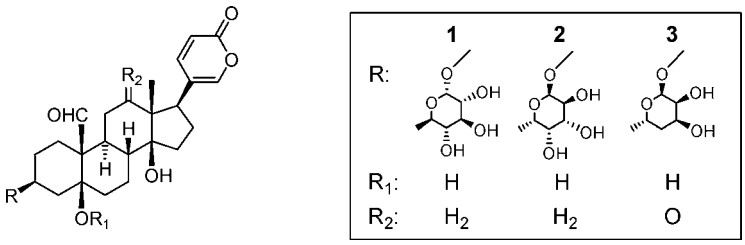
ABSTRACT



Three new compounds, kalanchosides A–C (1–3), as well as five known compounds, were isolated from the aerial parts of *Kalanchoe gracilis*. The compound structures were determined by spectroscopic methods. All eight isolated compounds showed significant cytotoxic activity against a panel of human tumor cell lines, with potency reaching the nanomolar range. However, only bryophyllin B (8) inhibited HIV replication in H9 lymphocyte cells.

Kalanchoe gracilis Hance (Crassulaceae) is a perennial, succulent medicinal herb grown in rocky terrain and indigenous to low altitudes of Taiwan.[1] It has been used as a Chinese medicine for the treatment of inflammation in wounds and bruises. The genus *Kalanchoe* is reported to contain bufadienolides,[2−5] fatty acids,[6] triterpenoids,[7] and flavonoids,[8−10] and to possess biological activities, such as blocking human lymphocyte proliferation[6,7] and inhibiting cancer cell growth.[4,11] However, thus far, only flavonoids have been reported from *K. gracilis*.[9,10] Our preliminary screening in the course of a continuing search for novel cytotoxic compounds from medicinal plants of Taiwan

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showed that the crude MeOH extract of *K. gracilis* exhibited significant cytotoxic activity against gastric and nasopharyngeal carcinoma cell lines. However, the reported flavonoids are unlikely to be responsible for such strong anticancer activity. Subsequent cytotoxicity assay guided fractionation and separation of the defatted MeOH extract of aerial parts of *K. gracilis* resulted in the isolation and identification of eight bufadienolides, including three new compounds, kalanchosides A (**1**), B (**2**), and C (**3**), as well as five known compounds, thesiuside (**4**),[12] hellebrigenin (**5**),[13] hellebrigenin-3-acetate (**6**),[2] bryophyllin A (**7**),[3] and bryophyllin B (**8**)[19] (Figure 1). Herein we describe the structural elucidation of the new bufadienolides and in vitro biological evaluation of cytotoxic and anti-HIV activities.

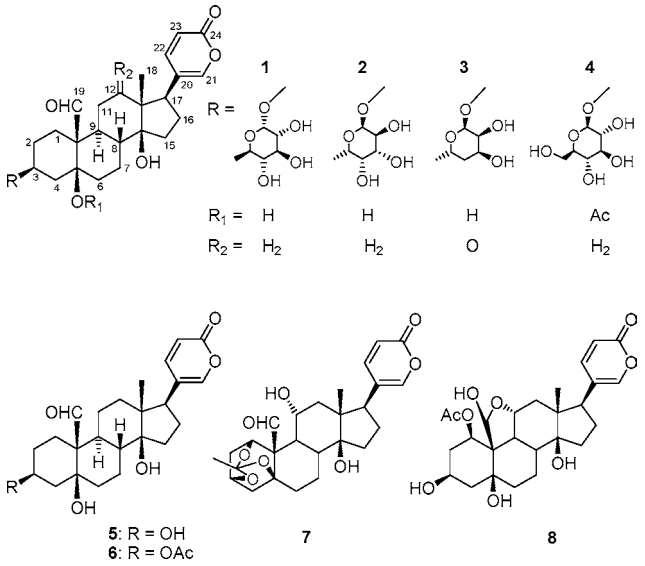

Figure 1. The structures of compounds **1**−**8**.

Kalanchoside A (**1**) was obtained as white amorphous powder. Its HR-FABMS revealed a *pseudo*-molecular ion at *m*/*z* 563.2853 $[M + H]^+$, corresponding to the molecular formula $C_{30}H_{42}O_{10}$. The presence of a 2-pyrone system was deduced from a UV absorption at 295 nm, a strong carboxyl IR absorption at 1709 cm^{-1}, and characteristic NMR signals (acetone-d_6, 300 MHz) at δ_H 6.17 (1H, d, $J = 9.8$ Hz, H-23), 7.40 (1H, d, $J = 1.6$ Hz, H-21), 7.95 (1H, dd, $J = 9.8$, 1.6 Hz, H-22) and δ_C 115.3 (C-23), 123.4 (C-20) 147.8 (C-22), 150.0 (C-21), 162.0 (C-24).[3] The NMR spectra also showed an aldehyde signal at δ_H 10.07 (s, H-19) and δ_C 208.5 (C-19), a tertiary hydroxyl signal at δ_H 3.43 (s, OH-14) and δ_C 84.9 (C-14), and a tertiary methyl signal at δ_H 0.69 (s, H-18) and δ_C 16.9 (C-18). Thus, compound **1** was identified as a bufadienolide. The ^{13}C NMR spectrum of the genin part of **1** was very similar to that of hellebrigenin [(3β,5,14)-trihydroxy-19-oxo-5β,14β-bufa-20,22-dienolide, **5**], which was also isolated from this plant. In addition, compound **1** had one sugar unit attached to the genin, which was confirmed by the presence of one anomeric proton at δ 4.87. The glycosyl moiety was identified as 6-deoxy-α-glucopyranoside by a three-proton doublet signal at δ 1.18 (3H, d, $J = 6.3$ Hz, H-6′), which was associated with the methyl carbon signal at δ 18.2 (C-6′) and five other methine signals at δ_H 2.99 (1H, t, $J = 9.3$ Hz, H-4′), 3.44 (1H, dd, $J = 9.3$, 3.7 Hz, H-2′), 3.60 (1H, t, $J = 9.3$ Hz, H-3′), 3.63 (1H, dq, $J = 9.3$, 6.3 Hz, H-5′), 4.87 (1H, d, $J = 3.7$ Hz, H-1′) and δ_C 69.0 (C-5′), 73.0 (C-2′), 74.7 (C-3′), 76.9 (C-4′), 97.3 (C-1′).[14] Diaxial coupling constants ($J = 9.3$ Hz) found between sugar protons (H-2′−H-5′) together with key NOE correlations between H-4′ and H-2′/H-6′ were consistent with a deoxy-α-D-glucose unit. An HMBC correlation between H-1′ (δ 4.87) and C-3 (δ 72.17) and a NOE correlation between H-1′ and H-3 established that the glucose moiety was linked to C-3 of the aglycon. The structure of **1** was thus established as hellebrigenin-3-*O*-6′-deoxy-α-glucopyranoside and named kalanchoside A.

Kalanchoside B (**2**) was isolated as amorphous powder and, with a molecular formula of $C_{30}H_{42}O_{10}$, was likely an isomer of **1**. Comparison of the ^1H and ^{13}C NMR spectra of **2** and **1** implied that the genin moieties were the same, but the sugar units were different. Rather than signals for a glucosyl moiety, signals assignable to an α-fucopyranosyl residue were observed in **2** at δ_H 1.18 (3H, d, $J = 6.4$ Hz, H-6′), 3.68 (1H, br s, H-4′), 3.76 (2H, m, H-2′ and -3′), 3.97 (1H, br q, $J = 6.4$ Hz, H-5′), 4.88 (1H, d, $J = 3.2$ Hz, H-1′) and δ_C 16.9 (C-6′), 67.5 (C-5′), 69.3 (C-2′), 71.2 (C-3′), 72.8 (C-4′), 97.7 (C-1′). The very small coupling between H-4′ and H-5′ and NOE correlations between H-5′ and H-4′/H-3′ were compatible with the stereochemistry of this group.[15] A HMBC correlation between H-1′ (δ 4.88) and C-3 (δ 71.9) and a NOE correlation between H-1′ and H-3 confirmed that the fucosyloxy moiety was attached at C-3. These data confirmed the structure of hellebrigenin-3-*O*-α-fucopyranoside for kalanchoside B (**2**).

Kalanchoside C (**3**), white amorphous powder, has the molecular formula $C_{30}H_{40}O_{10}$ based on HR-FABMS. The UV, IR, and NMR spectra showed the characteristic features of a bufadienolide. The ^1H and ^{13}C NMR spectral data of the genin moiety of **3** were quite similar to those of **1** and **2**, except for ring C, where a ketonic group was observed at δ_C 211.8 rather than a methylene group.

The keto functionality was located at C-12, due to the downfield shifts of H-11 (δ 2.27, dd, $J = 14.0$, 4.2 Hz and 2.59, t, $J = 14.0$ Hz) and H-17 (δ 3.95, m), as well as C-11 (δ 38.3) and C-13 (δ 64.0). The glycoside moiety of **3** was determined to be a 4,6-dideoxy-α-*ribo*-hexopyranose based on ^1H and ^{13}C signals at δ_H 1.11 (3H, d, $J = 6.3$ Hz, H-6′), 3.53 (1H, dt, $J = 8.7$, 3.9 Hz, H-2′), 3.88 (1H, d, $J = 8.7$ Hz, OH-2′), 3.95 (1H, m, H-3′), 4.25 (1H, m, H-5′), 4.85

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Table 1. Cytotoxicity Data for **1**−**8** against a Human Tumor Cell Line Panel[a]

compd	IC_{50} (μg/mL) for 3 days continuous exposure						
	KB	KB−VIN	A549	1A9	PC-3	HCT-8	A431
1	0.003	0.003	0.0005	0.0008	0.002	0.006	0.007
2	0.005	0.013	0.001	0.007	0.010	0.015	0.022
3	0.016	0.026	0.006	0.012	0.025	0.045	0.055
4	0.006	0.006	0.0005	0.002	0.006	0.010	0.014
5	0.007	0.007	0.001	0.003	0.008	0.010	0.025
6	0.008	0.008	0.055	0.003	0.012	0.015	0.028
7	0.1	0.025	0.013	0.014	0.030	0.060	0.3
8	0.6	0.5	0.07	0.2	0.8	0.8	1.4
etoposide	0.7	7.2	1.0	0.14	4.7	ND	ND
vincristine	0.004	1.0	ND[b]	ND	ND	ND	ND
vincristine + verapamil	ND	0.004	ND	ND	ND	ND	ND

[a] Cell lines: KB = nasopharyngeal; KB-VIN = MDR subline; A549 = lung; 1A9 = ovarian; PC-3 = prostate; HCT-8 = ileocecal; A431 = epidermoid. [b] ND = not determined.

(1H, d, J = 3.9 Hz, H-1′) and δ_C 21.0 (C-6′), 40.4 (C-4′), 60.3 (C-5′), 68.3 (C-3′), 68.7 (C-2′), 100.7 (C-1′).[16] The NOE correlations of H-4′ax (δ 1.51) with H-2′, -3′, and -6′ and of H-4′eq (δ 1.83) with H-5′ and -6′ revealed that H-2′ has an axial orientation while H-1′ and -3′ are directed equatorially. The small coupling constant (3.9 Hz) between H-2′ and H-1′, -3′ further confirmed the stereochemistry of the sugar moiety. Therefore, kalanchoside C (**3**) was assigned as 12-oxo-hellebrigenin-3-*O*-4,6-dideoxy-α-*ribo*-hexopyranoside.

It has been proposed that a bufadienolide has a four-ring structure with rings A, B, C, and D generally in a *cis*/*trans*/*cis* configuration and chair/chair/chair conformation.[5] This proposal can be supported by NOE experiments. For example, kalanchoside A (**1**), H-18 [δ 0.69 (3H, s)] exhibited NOE correlations with H-8 [δ 1.96 (1H, td, J = 16.0, 3.0 Hz)] and H-11β (δ 1.16); H-19 [δ 10.07 (1H, s)] showed NOE coupling with H-6β (δ 2.17), H-8, and H-11β; and H-3 [δ 4.07 (1H, br s)] presented NOE cross peaks with two H-2 and H-4 protons (Figure 2). However, a chair/boat/chair conformation was found in the structure of bryophyllin B (**8**). First, H-11 must be oriented axially on the basis of its NOE correlations with H-8 [δ 2.50 (1H, td, J = 11.5, 2.5 Hz)] and H-18 [δ 0.86 (3H, s)]. Second, H-19 has an α-orientation due to the NOE correlation between H-19 [δ 5.79 (1H, d, J = 4.8 Hz)] and H-2β (δ 2.07). Finally, other NOE correlations, including H-6α (δ 1.95) with H-1 [δ 4.65 (1H, dd, J = 12.6, 3.7 Hz)], H-3 [δ 3.81 (1H, br s)], and H-7α (δ 1.18); H-9 [δ 1.29 (1H, t, J = 11.5 Hz)] with H-1 and H-12α (δ 1.46), as well as H-3 with H-2α (δ 1.50) and H-4α (δ 1.89), revealed that the B-ring adopts a boat conformation in order to form the fused hemiacetal ring (Figure 2).

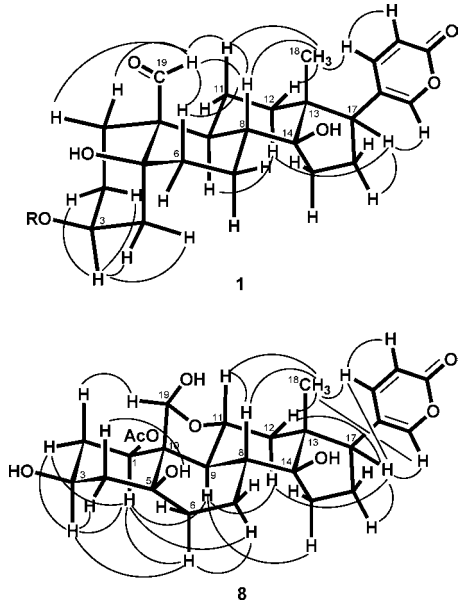

Figure 2. NOE correlations of **1** and **8**.

Bufadienosides **1**−**8** were assayed for anti-HIV activity in H9 lymphocyte cells according to a literature method.[17,18] Only bryophyllin B (**8**) inhibited HIV replication with an ED_{50} value of <0.25 μg/mL and therapeutic index of >6.27. The remaining compounds were toxic at all tested concentrations (25, 2.5, 0.25 μg/mL). In contrast, all eight compounds showed significant, broad spectrum cytotoxic activity against human tumor cell lines [nasopharyngeal (KB), its MDR variant (KB-VIN), lung (A549), ovarian (1A9), prostate (PC-3), ileocecal (HCT-8), and epidermoid (A431)] (Table 1) as described in the literature.[18]

Compounds **1** and **4** showed remarkable potency against the A549 cell line with an IC_{50} value of 0.5 ng/mL. IC_{50} values for these two compounds against the other tested cell lines ranged from 2 to 14 ng/mL. Therefore, converting the C-6′ methyl to a hydroxymethyl did not appear to significantly affect the cytotoxicity in these cell lines.

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However, there was a notable drop in cytotoxicity for kalanchosides B (**2**) and C (**3**), although compounds **2**, **3**, **5**, and **6** showed significant potencies ranging from 1 ng/mL to 0.055 μg/mL. Compound **7** was slightly less potent (IC_{50} 0.013−0.3 μg/mL), and compound **8** was least potent (IC_{50} 0.07 to 1.4 μg/mL). The weaker activity of **8** is likely due to the removal of the aldehyde moiety.

Overall, except for compound **8**, all others displayed strong potency in this cell line panel, including KB-VIN, a drug-resistant cell line. In general, these active compounds were not substrates for P-glycoprotein based on similar potency in KB vs KB-VIN cell lines. These results nonetheless suggest that bufadienolides could serve as potential leads and templates for further SAR studies as anticancer drug candidates.

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Note Added after ASAP Publication. Bryophyllin B (**8**) was incorrectly identified as kalanchoside D (**4**) in the version published ASAP September 15, 2006. Text describing this compound, reference 19, and revised Supporting Information are included in the revised version published ASAP October 12, 2006.

Supporting Information Available: Isolation procedures and full characterization data. This material is available free of charge via the Internet at http://pubs.acs.org.

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