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Cytotoxic bufadienolides from the whole plants of *Helleborus foetidus*

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ABSTRACT

Two new bufadienolides (**1** and **2**) and three known bufadienolide glucosides (**3**–**5**) were isolated from the whole plants of *Helleborus foetidus* (Ranunculaceae). The structures of **1** and **2** were determined spectroscopically. Compound **1** displayed potent cytotoxicity against HL-60 and A549 cells, with IC_{50} values of 0.035 and 0.029 μM, respectively. HL-60 cells treated with **1**displayed typical characteristics of apoptosis, such as nuclear chromatin condensation, DNA fragmentation, accumulation of sub-G1 cells, and marked activation of caspase-3. Moreover, the loss of mitochondrial membrane potential and release of cytochrome *c* into the cytosol suggested that **1** induced HL-60 cell death via the mitochondrial-dependent apoptotic pathway.

1. Introduction

The genus *Helleborus* belongs to the family Ranunculaceae, which has approximately 20 species (Tsukamoto, 1989). We have previously studied the chemical components of the rhizomes of *Helleborus orientalis* and achieved the isolation of a variety of steroidal components (Mimaki et al., 2003, 2010; Watanabe et al., 2002, 2003, 2005). *Helleborus foetidus* L. is an evergreen perennial plant, native to Europe. Although anemonin, a quercetin glycoside, a furostan glycoside, and a few phenolic glucosides were isolated from *H. foetidus*, there have been no systematic phytochemical investigations or evaluations of biological activity with regard to the secondary metabolites of this plant (Prieto et al., 2006). Therefore, the present study aimed to examine the cytotoxicity of the chemical components of *H. foetidus* on two cancer cell lines, namely HL-60 and A549 cells. The cytotoxicity-guided fractionation of MeOH extract of the whole plants of *H. foetidus* isolated two new bufadienolides (**1** and **2**) and three known bufadienolide glucosides. The present report details the isolation and structural determination of the two new bufadienolides (**1** and **2**), the evaluation of the cytotoxicity of the isolated compounds, and the apoptosis-inducing activity of **1**.

2. Results and discussion

Following the extraction and isolation procedure described in section 3.3, compounds **1**–**5** were obtained. On the basis of their physical and spectroscopic data, compounds **3**–**5** were identified as 11α-hydroxyscilliglaucoside (**3**) (Krenn et al., 2000), desacetylscillicyanosid (**4**) (Lichti et al., 1973), and hellebortin A (**5**) (Meng et al., 2001) (Fig. 1).

Compound **1** was isolated as an amorphous solid with the molecular formula $C_{24}H_{30}O_7$, as determined by the data of HR-ESI-TOF-MS (*m/z* 453.1907 $[M + Na]^+$) and ^{13}C NMR. The UV spectrum of **1** showed absorption maxima at 296 and 206 nm owing to a conjugated system. The IR spectrum suggested the presence of hydroxy groups at 3401 cm^{-1} and carbonyl groups at 1713 and 1698 cm^{-1}. The ^1H NMR spectrum contained signals for a 2-pyrone ring moiety at δ_H 8.08 (1H, dd, J = 9.7, 2.5 Hz, H-22), 7.47 (1H, br d, J = 2.5 Hz, H-21), and 6.19 (1H, br d, J = 9.7 Hz, H-23), which were characteristic of the bufadienolide structure, with two olefinic protons at δ_H 5.95 (1H, br d, J = 9.9 Hz, H-3) and 5.42 (1H, d, J = 9.9 Hz, H-4), two hydroxymethine protons at δ_H 4.45 (1H, ddd, J = 7.2, 7.2, 1.1 Hz, H-16) and 3.70 (1H, ddd, J = 10.1, 9.6, 4.1 Hz, H-11), an aldehyde group at δ_H 9.90 (1H, s, H-19), and a methyl group at δ_H 0.81 (3H, s, H-18). The ^{13}C NMR spectrum revealed a 2-pyrone ring moiety at δ_C 120.0, 151.9, 152.3, 113.2, and 120.0 (C-20–C-24), an aldehyde carbon at δ_C 210.3 (C-19), a set of olefinic carbons at δ_C 132.4 (C-3) and 133.6 (C-4), two quaternary carbons with an oxygen atom at δ_C 84.9 (C-14) and 72.6 (C-5), and two quaternary carbons at δ_C 54.7 (C-10) and 50.3 (C-13). Analysis of the ^1H-^1H COSY and HMQC spectra of **1** revealed that **1** had four proton spin systems, a: $-C_{(1)}H_2-C_{(2)}H_2-C_{(3)}H=C_{(4)}H-$, b: $-C_{(6)}H_2-C_{(7)}H_2-C_{(8)}H-C_{(9)}H-C_{(11)}H(-OH)-C_{(12)}H_2-$, c: $-C_{(15)}H_2-C_{(16)}H(-OH)-C_{(17)}H-$, and d: $-C_{(22)}H=C_{(23)}H-$. In the HMBC spectrum, long range correlations were observed between δ_H 9.90 (H-19) and δ_C 23.6 (C-1)/45.6 (C-9)/54.7 (C-10), δ_H 5.42 (H-4) and δ_C 37.9 (C-6)/54.7 (C-10), δ_H 0.81 (CH_3-18) and δ_C 50.5 (C-12)/84.9 (C-14)/57.9 (C-17), δ_H 3.70 (H-11)/1.39 (H-12b)/2.87 (H-17) and δ_C 50.3 (C-13), and δ_H 1.98 (H-8)/1.47(H-9)/1.65 (H-15b)/4.45 (H-16) and δ_C 84.9 (C-14), as shown in Fig. 2. Therefore, **1** was a bufadienolide with

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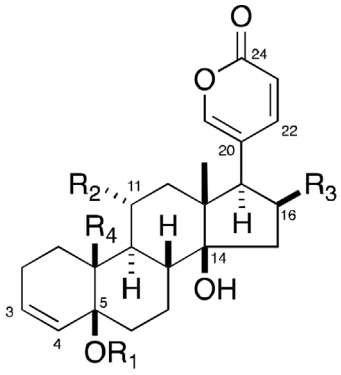

	R_1	R_2	R_3	R_4
1	H	OH	OH	CHO
2	H	H	OH	OH
3	Glc	H	OH	OH
4	Glc	OH	H	CHO
4a	H	OH	H	CHO
5	Glc	H	OH	CHO
5a	H	H	OH	CHO

Glc= β-D-glucopyranosyl

Fig. 1. Structures of **1**–**5**, **4a**, and **5a**.

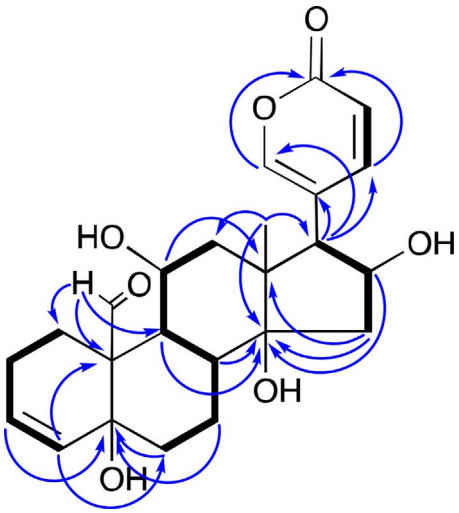

Fig. 2. Key HMBC correlations of **1**.
Bold lines indicate the ^{1}H-^{1}H coupling, and arrows indicate ^{1}H/^{13}C long-range correlations.

an aldehyde group at C-19 and four hydroxy groups at C-5, C-11, C-14, and C-16. Furthermore, the NOE correlations between H-4 and H-6eq, H-2α and H-9, and H-12ax and H-17 observed in the phase-sensitive NOESY spectrum (Fig. 3) and the proton spin-coupling constants (Table 1) indicated that **1** had A/B *cis*, B/C *trans*, and C/D *cis* ring junctions and the 11α and 16β configurations. Thus, the structure of **1** was determined as 5β,11α,14β,16β-tetrahydroxy-19-oxobufa-3,20,22-trienolide.

Compound **2** was obtained as an amorphous solid with the molecular formula $C_{23}H_{30}O_6$, as determined by HR-ESI-TOF-MS (*m/z* 425.1934 $[M + Na]^+$). The ^{1}H and ^{13}C NMR spectra of **2** were very similar to the aglycone moiety of **3**. However, the resonances of the β-D-

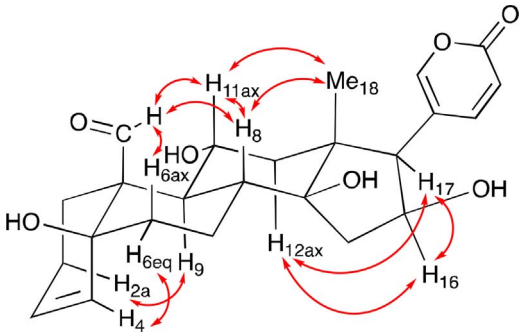

Fig. 3. Key NOE correlations of **1**.

Table 1
^{1}H and ^{13}C NMR chemical shift assignments of **1** and **2**[a].

1			**2**		
	^{1}H	^{13}C		^{1}H	^{13}C
1a	2.57	23.6	1a	1.87	28.1
b	2.16		b	1.72	
2a	2.25	23.0	2a	2.13	25.9
b	2.11		b	2.06	
3	5.95 br d (9.9)[b]	132.4	3	5.83 br d (9.6)	132.1
4	5.42 d (9.9)	133.6	4	5.54 d (9.6)	133.6
5	–	72.6	5	–	74.6
6a	1.95	37.9	6a	1.87	36.1
b	1.73		b	1.59	
7a	2.09	25.0	7a	1.97	25.0
b	1.11		b	0.97	
8	1.98	42.3	8	1.68	41.6
9	1.47	45.6	9	1.38	41.1
10	–	54.7	10	–	72.1
11	3.70 ddd (10.1, 9.6, 4.1)	68.8	11a	1.58	22.7
			b	1.48	
12a	1.64	50.5	12a	1.59	42.0
b	1.39		b	1.36	
13	–	50.3	13	–	50.2
14	–	84.9	14	–	85.6
15a	2.35	42.5	15a	2.33	42.9
b	1.65		b	1.65	
16	4.45 ddd (7.2, 7.2, 1.1)	73.7	16	4.46 ddd (7.7, 7.7, 0.9)	73.5
17	2.87 d (7.2)	57.9	17	2.75 d (7.7)	59.3
18	0.81 s	19.3	18	0.81 s	17.2
19	9.90 s	210.3	19	–	
20	–	120.0	20	–	120.5
21	7.47 br d (2.5)	151.9	21	7.44 dd (2.5, 0.8)	151.7
22	8.08 dd (9.7, 2.5)	152.3	22	8.12 dd (9.7, 2.5)	152.9
23	6.19 br d (9.7)	113.2	23	6.19 dd (9.7, 0.8)	112.9
24	–	164.9	24	–	165.1

[a] Compound **1** and **2** were measured in CD_3OD.
[b] Values in parentheses are coupling constants in Hz.

glucopyranosyl moiety were not observed in the spectra of **2**. The molecular formula of **2** was lower than that of **3** by a mass equivalent to $C_6H_{11}O_5$. The enzymatic hydrolysis of **3** by using naringinase produced **2** and D-glucose. Thus, the formulation of **2** was determined as 5β,10β,14β,16β-tetrahydroxy-19-norbufa-3,20,22-trienolide.

The isolated compounds (**1**–**5**) and the aglycones (**4a** and **5a**) were evaluated for their cytotoxic activity against HL-60 and A549 cells. All compounds exhibited cytotoxic activity against both HL-60 and A549 cells; the IC_{50} values were in the range from 0.0029–3.6 μM (Table 2). Compounds **3**–**5** were bufadienolide glucosides that also exhibited cytotoxicity against HL-60 and A549 cells (IC_{50} values for **3**: 2.7 and 1.2 μM; **4**: 0.0076 and 0.0029 μM; and **5**: 0.025 and 0.012 μM, respectively). The cytotoxicity of **2**, **4a**, and **5a**, which were the aglycones of **3**–**5**, were almost equal to that of **3**–**5** (IC_{50} values for **2**: 3.6 and 1.2 μM; **4a**: 0.011 and 0.0040 μM; and **5a**: 0.034 and 0.015 μM, respectively). These results showed that the glucosyl group at the C-5 of