Journal of Natural Medicines (2021) 75:393–402
https://doi.org/10.1007/s11418-021-01481-6

ORIGINAL PAPER

Bufadienolides and ecdysteroids from the whole plants of *Helleborus niger* and their cytotoxicity

Akihito Yokosuka[1] · Mina Inomata[1] · Yuka Yoshizawa[1] · Tomoki Iguchi[1] · Yoshihiro Mimaki[1]

Received: 10 October 2020 / Accepted: 5 January 2021 / Published online: 27 January 2021

Abstract

A new bufadienolide (**1**), two new bufadienolide glycosides (**2** and **3**), a new ecdysteroid (**4**), and four known compounds (**5**–**8**), were isolated from the whole plants of *Helleborus niger* L. (Ranunculaceae). The structures of the new compounds (**1**–**4**) were determined by spectroscopic analysis, including 2D NMR spectral data, and hydrolytic studies. Compounds **1**–**6** showed cytotoxicity against HL-60 human leukemia cells, A549 human lung adenocarcinoma cells, and SBC-3 human small-cell lung cancer cells, with IC_{50} values ranging from 0.0055 to 1.9 μM. HL-60 cells treated with either **3** or **4** showed apoptosis characteristics, such as nuclear chromatin condensation, accumulation of sub-G_1 cells, and activation of caspase-3/7.

Keywords *Helleborus niger* · Ranunculaceae · Bufadienolides · Ecdysteroids · Cytotoxicity · HL-60 cells · A549 cells · SBC-3 cells · Apoptosis

Introduction

The genus *Helleborus* (Ranunculaceae) includes approximately 20 plant species [1], most of which are cultivated for ornamental purposes. *Helleborus* plants are commonly called Christmas rose, and are known to be poisonous because they contain cardiotonic steroidal components such as bufadienolides [2–4]. Previously, we have examined the chemical constituents of three *Helleborus* species, *H. orientalis*, *H. foetidus*, and *H. lividus*, and characterized steroidal compounds, including bufadienolides, bufadienolide glycosides, and spirostanol glycosides [5–9].

As part of our ongoing phytochemical studies of steroidal components from *Helleborus* plants, we investigated the whole plants of *H. niger*, which have been used as a medicinal herb for laxative and anthelmintic purposes [10]. Although a few steroidal glycosides and flavonoids have been isolated from *H. niger* [11, 12], there have been no systematic phytochemical studies or evaluations of biological activities associated with the secondary metabolites of this plant.

In the present study we carried out phytochemical analysis of the MeOH extract of *H. niger*, which resulted in the isolation of a new bufadienolide, two new bufadienolide glycosides, a new ecdysteroid, and four known compounds. The isolated compounds were evaluated for their cytotoxic activities against HL-60 human promyelocytic leukemia cells, A549 human lung adenocarcinoma cells, and SBC-3 human small-cell lung cancer cells. The apoptosis-inducing activities of **3** and **4** against HL-60 cells were also investigated.

Results and discussion

The whole plants of *H. niger* (4.5 kg) were extracted with MeOH. The MeOH extract, which showed cytotoxicity against HL-60, A549, and SBC-3 cells with IC_{50} values of 2.6, 1.8, and 1.9 μg/mL, respectively, was subjected to a porous-polymer polystyrene resin (Diaion HP-20) column, and successively eluted with 30% MeOH, 50% MeOH, MeOH, EtOH, and EtOAc. The 50% MeOH eluate was cytotoxic to HL-60 cells (IC_{50} 0.37 μg/mL), A549 cells (IC_{50} 0.42 μg/mL), and SBC-3 cells (IC_{50} 0.25 μg/mL). The 50% MeOH eluate was subjected to multiple chromatographic

Supplementary Information The online version contains supplementary material available at https://doi.org/10.1007/s11418-021-01481-6.

✉ Akihito Yokosuka
yokosuka@toyaku.ac.jp

[1] Department of Medicinal Pharmacognosy, School of Pharmacy, Tokyo University of Pharmacy and Life Sciences, 1432-1 Horinouchi, Hachioji, Tokyo 192-0392, Japan

steps over silica gel and octadecylsilanized (ODS silica gel to give **1**–**8** (Fig. 1).

Compound **1** was obtained as an amorphous solid with a molecular formula of $C_{24}H_{30}O_6$ as determined from its HR-ESI/TOF-MS (*m/z*: 437.1937 $[M+Na]^+$) and ^{13}C NMR spectral data. The IR spectrum of **1** showed absorption bands for hydroxy groups (3358 cm^{-1}) and carbonyl groups (1712 and 1650 cm^{-1}). The UV spectrum showed the absorption maxima indicative of a conjugated system (293.5 and 205.5 nm). The ^1H and ^{13}C NMR spectra of **1** contained signals for a 2-pyrone ring moiety characteristic of the bufadienolide skeleton [δ_H 8.12 (dd, $J=9.7$, 2.3 Hz, H-22), 7.45 (br d, $J=2.3$ Hz, H-21), 6.20 (br d, $J=9.7$ Hz, H-23)/δ_C 120.3, 151.8, 152.7, 113.0, and 165.0 (C-20–C-24)]; an olefinic group [δ_H 5.90 (s, H-4)/δ_C 171.3 (C-5) and 126.7 (C-4)]; a tertiary methyl group [δ_H 0.83 (s, Me-18)/δ_C 17.3 (C-18)]; an oxymethine group [δ_H 4.48 (br dd, $J=8.0$, 8.0 Hz, H-16)/δ_C 73.3 (C-16)]; a hydroxymethyl group [δ_H 3.99 (d, $J=11.1$ Hz, H-19a) and 3.81 (d, $J=11.1$ Hz, H-19b)/δ_C 65.9]; a quaternary carbon with an oxygen atom [δ_C 85.5 (C-14)]; two quaternary carbons [δ_C 50.3 (C-13) and 45.2 (C-10)]; and an carbonyl carbon [δ_C 203.1 (C-3)] (Table 1). Detailed spectral analysis of the ^1H–^1H COSY and HSQC spectra of **1** indicated that the structure of **1** was composed of four spin-coupling systems (fragments A–D), corresponding to the following structural fragments; A: H_2-1/H_2-2, B: H_2-6/H_2-7/H-8/H-9/H_2-11/H_2-12, C: H_2-15/H-16/H-17, and D: H-22/H-23 (Fig. 2). In the HMBC spectrum of **1**, the hydroxymethyl protons at δ_H 3.99 and 3.81 showed long-range correlations with C-1 (δ_C 34.3), C-5 (δ_C 171.3), C-9 (δ_C 50.9), and C-10 (δ_C 45.2), and was assigned to H_2-19. In the HSQC spectrum, the C-1 carbon was correlated to the one-bond coupled protons at δ_H 2.42 and 1.80 (each m, H_2-1), which showed spin-coupling correlations with the methylene protons at δ_H 2.73 and 2.29 (each m, H_2-2) in the ^1H–^1H COSY spectrum. The H_2-1 and H_2-2 protons displayed HMBC correlations with the C-3 carbonyl carbon at δ_C 203.1 and C-10. The olefinic proton at δ_H 5.90 attributed to H-4 exhibited $^3J_{C,H}$ correlations with C-6 (δ_C 35.0) and C-10. These correlations were indicative of linkage of fragments A and B through the C-10 quaternary carbon, and the locus of a carbonyl carbon at C-3 and a double-bond between C-4 and C-5. The angular methyl singlet at δ_H 0.83 assigned to Me-18 showed HMBC correlations with C-12 (δ_C 42.1), C-13 (δ_C 50.3), C-14 (δ_C 85.5), and C-17 (δ_C 59.3), which implied that fragment B was linked to fragment C through the C-13 quaternary carbon and C-14 quaternary carbon having a hydroxy group. In the ^1H–^1H COSY spectrum, the hydroxymethine proton at δ_H 4.48 showed spin-coupling correlations with the methylene protons at δ_H 2.44 (dd, $J=14.6$, 8.0 Hz, H-15α) and 1.72 (d, $J=14.6$ Hz, H-15β) and the methine proton at δ_H 2.77 (d, $J=8.0$ Hz, H-17), and assigned to H-16. The 2-pyrone ring was confirmed to be attached to C-17 based on HMBC correlations from H-17 (δ_H 2.77) to C-20 (δ_C 120.3), C-21 (δ_C 151.8), and C-22 (δ_C 152.7) (Fig. 2). Therefore, the

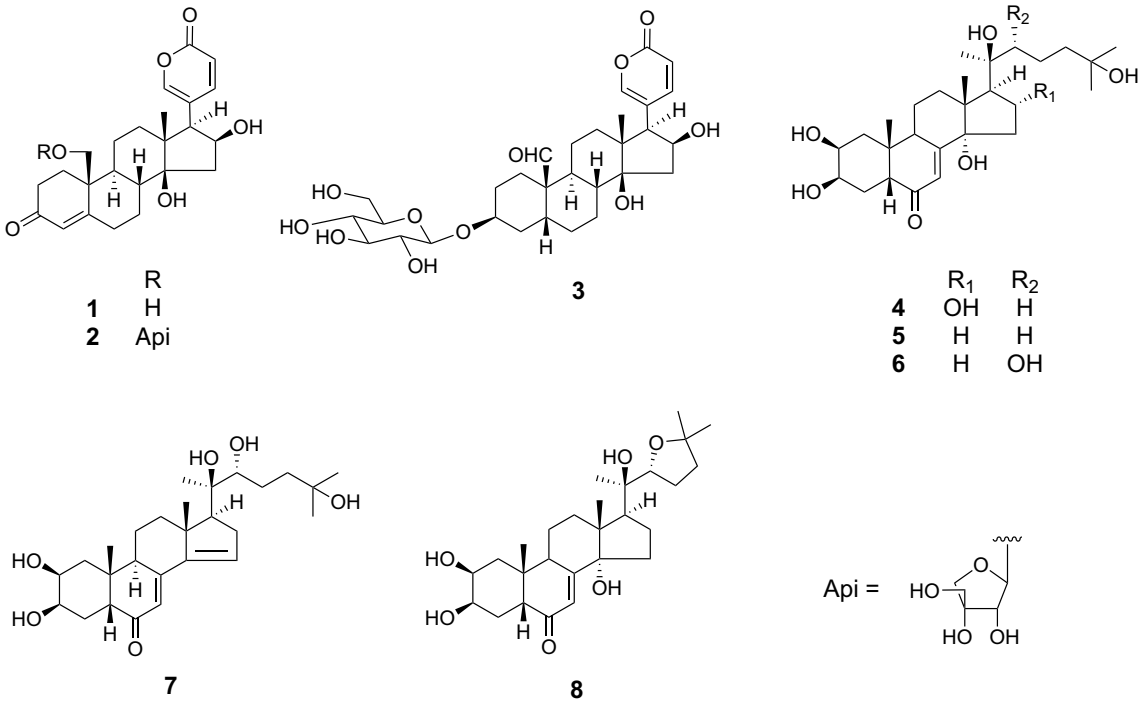


Fig. 1 Chemical structures of **1**–**8**

Table 1 ^{1}H (600 MHz for **1**, **2**, **3**, and **3a**; 500 MHz for **4**) and ^{13}C (150 MHz for **1**, **2**, **3**, and **3a**; 125 MHz for **4**) NMR chemical shift assignments of **1–3**, **3a**, and **4** in CD_3OD

Position		**1**		Position		**2**		Position		**3**		**3a**		Position		**4**	
		^{1}H	^{13}C			^{1}H	^{13}C			^{1}H	^{13}C	^{1}H	^{13}C			^{1}H	^{13}C
1	*α*	2.42 m	34.3	1	*α*	2.42 m	34.8	1	*α*	1.82 m	22.6	1.77 m	22.2	1	*α*	1.79 m	37.3
	β	1.80 m			*β*	1.83 m			*β*	1.56 m		1.54 m			*β*	1.43 m	
2	*α*	2.73 m	35.7	2	*α*	2.70 m	35.7	2	*α*	1.85 m	26.0	1.60 m	27.0	2		3.84 m	68.5
	β	2.29 m			*β*	2.29 m			*β*	1.60 m		1.59 m					
3		–	203.1	3		–	203.0	3		4.12 br s	74.3	4.08 br s	66.5	3		3.95 m	68.7
4		5.90 s	126.7	4		5.84 s	126.4	4	*α*	1.84 m	29.9	1.94 m	33.2	4	*α*	1.77 m	32.8
									β	1.71 m		1.48 m			*β*	1.74 m	
5		–	171.3	5		–	170.9	5		2.35 m	30.6	2.29 m	30.4	5		2.38 dd (9.4, 4.4)	51.8
6	*α*	2.51 m	35.0	6	*α*	2.50 m	34.9	6	*α*	1.59 m	29.3	1.61 m	29.3	6		–	206.3
	β	2.42 m			*β*	2.42 m			*β*	1.44 m		1.40 m					
7	*α*	2.35 m	29.4	7	*α*	2.35 m	29.3	7	*α*	1.90 m	22.7	1.91 m	22.6	7		5.77 s	122.2
	β	1.25 m			*β*	1.24 m			*β*	1.32 m		1.27 m					
8		1.87 m	43.4	8		1.88 m	43.5	8		1.85 m	43.2	1.82 m	43.2	8		–	166.9
9		1.34 m	50.9	9		1.35 m	51.0	9		1.80 m	36.0	1.80 m	35.9	9		3.18 dd	34.9
10		–	45.2	10		–	43.8	10		–	52.2	–	52.3	10		–	39.3
11	*α*	1.62 m	22.8	11	*α*	1.63 m	22.8	11	*α*	1.80 m	22.0	1.75 m	21.9	11	*α*	1.80 m	21.1
	β	1.44 m			*β*	1.42 m			*β*	1.47 m		1.48 m			*β*	1.68 m	
12	*α*	1.62 m	42.1	12	*α*	1.63 m	41.9	12	*α*	1.59 m	42.0	1.60 m	41.9	12	*α*	2.29 m	32.4
	β	1.34 m			*β*	1.35 m			*β*	1.38 m		1.38 m			*β*	1.80 m	
13		–	50.3	13		–	50.3	13		–	50.4	–	50.4	13		–	50.3
14		–	85.5	14		–	85.4	14		–	85.6	–	85.6	14		–	84.7
15	*α*	2.44 dd (14.6, 8.0)	42.8	15	*α*	2.44 dd (14.7, 7.6)	42.8	15	*α*	2.56 dd (14.7, 7.6)	42.6	2.55 dd (14.8, 7.8)	42.6	15	*α*	2.42 dd (14.1, 8.3)	43.0
	β	1.72 d (14.6)			*β*	1.72 d (14.7)			*β*	1.74 d (14.7)		1.74 d (14.8)			*β*	1.66 d (14.1)	
16		4.48 dd (8.0, 8.0)	73.3	16		4.48 dd (7.6, 7.6)	73.3	16		4.51 dd (7.6, 7.6)	73.4	4.50 dd (7.8, 7.8)	73.4	16		4.49 dd (8.3, 6.1)	72.6
17		2.77 d (8.0)	59.3	17		2.77 d (7.6)	59.2	17		2.77 d (7.6)	59.4	2.76 d (7.8)	59.4	17		2.33 d (6.1)	59.2
18		0.83 s	17.3	18		0.79 s	17.3	18		0.84 s	17.1	0.83 s	17.1	18		0.86 s	19.0
19	a	3.99 d (11.1)	65.9	19	a	4.12 d (9.5)	72.3	19		9.53 s	208.6	9.55 s	208.1	19		0.96 s	24.4
	b	3.81 d (11.1)			b	3.63 d (9.5)											
20		–	120.3	20		–	120.3	20		–	120.4	–	120.4	20		–	75.9
21		7.45 br d (2.3)	151.8	21		7.45 br d (2.5)	151.8	21		7.45 br d (2.0)	151.8	7.44 br d (1.7)	151.8	21		1.30 s	26.6
22		8.12 dd (9.7, 2.3)	152.7	22		8.12 dd (9.8, 2.5)	152.8	22		8.14 dd (9.7, 2.0)	152.9	8.14 dd (9.7, 1.7)	152.9	22	a	1.72 m	45.7
															b	1.53 m	
23		6.20 br d (9.7)	113.0	23		6.20 br d (9.8)	112.9	23		6.19 br d (9.7)	112.9	6.18 br d (9.7)	112.9	23	a	1.53 m	19.9
															b	1.45 m	
24		–	165.0	24		–	165.0	24		–	165.1	–	165.1	24	a	1.53 m	45.3
															b	1.45 m	

Table 1 (continued)

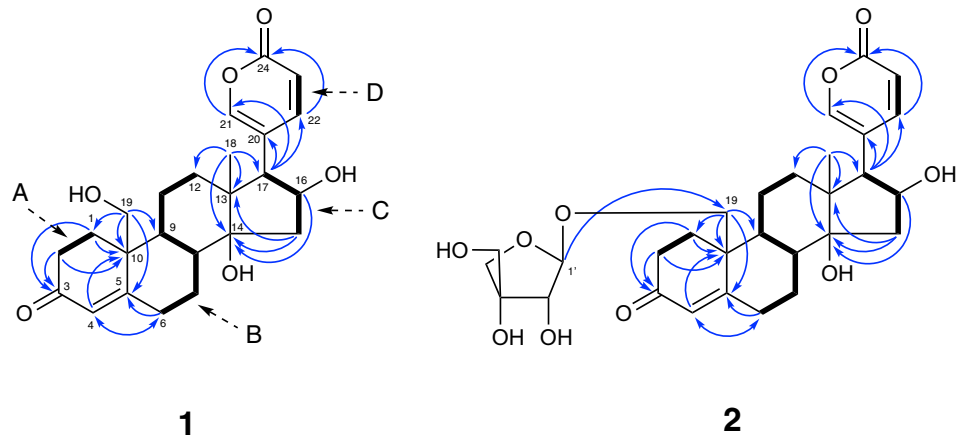
Position	**1**		Position	**2**		Position	**3**		**3a**		Position	**4**	
	^{1}H	^{13}C		^{1}H	^{13}C		^{1}H	^{13}C	^{1}H	^{13}C		^{1}H	^{13}C
											25	–	71.6
											26	1.19 s	29.2
											27	1.19 s	29.4
			Api			Glc							
			1′	4.89 d (2.3)	110.4	1′	4.35 d (7.8)	102.7					
			2′	3.77 d (2.3)	78.1	2′	3.20 dd (7.8, 7.8)	75.2					
			3′	–	80.4	3′	3.36 dd (7.8, 7.8)	78.2					
			4′	a 3.88 d (9.7)	75.2	4′	3.29 dd (7.8, 7.8)	71.5					
				b 3.75 d (9.7)		5′	3.26 m	77.9					
			5′	a 3.49 dd (11.4)	65.4	6′	a 3.85 dd (12.0, 2.2)	62.8					
				b 3.45 dd (11.4)			b 3.66 dd (12.0, 5.6)						

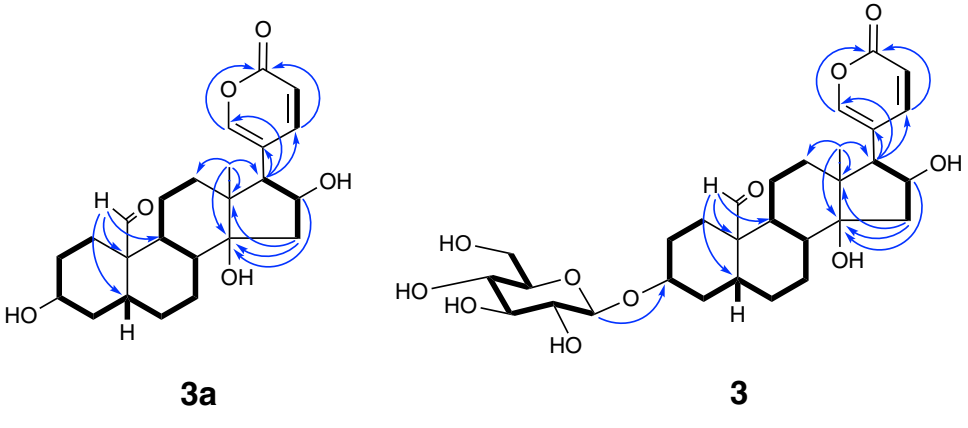
[a]Values in parentheses are coupling constants in Hz

structure of **1** was revealed to be a bufadienolide derivative with a carbonyl group at C-3, a double-bond between C-4 and C-5, and three hydroxy groups at C-14, C-16, and C-19. In the phase-sensitive NOESY (PHNOESY), NOE correlations between H-8 and H-11β/Me-18/H_2-19 indicated that **1** had the B/C *trans* ring fusion, and those between H-12β and H-16/H-17 showed that **1** had C/D *cis* ring fusion, and the 14β-OH, 16β-OH, and 17β-2-pyrone-ring (Fig. 3). Based on the above data, **1** was elucidated as 14β,16β,19-trihydroxy-3-oxobufa-4,20,22-trienolide.

It was suggested that compound **2** ($C_{29}H_{38}O_{10}$) was a glycoside of **1** based on the ^{1}H and ^{13}C NMR spectra, which showed anomeric proton and carbon signals at δ_H 4.89 (d, J = 2.3 Hz, H-1′) and δ_C 110.4 (C-1′). The molecular formula of **2** was larger than that of **1** by $C_5H_8O_4$, and acid hydrolysis of **2** with 0.5 M HCl gave **1** as the aglycone, and D-apiose. The identification of D-apiose was carried out by direct HPLC analysis of the hydrolysate using an optical rotation detector. In the ^{13}C NMR spectrum of **2**, a set of five signals assignable to a β-D-apiofuranosyl unit (Api) [δ_C 110.4, 78.1, 80.4, 75.2, and 65.4 (C-1′–C-5′)] were observed [13]. A $^{3}J_{C,H}$ correlation across the glycosidic linkage was detected between H-1′ of Api at δ_H 4.89 and C-19 of the aglycone at δ_C 72.3 in the HMBC spectrum (Fig. 2). Thus, **2** was established as 19-[(β-D-apiofuranosyl) oxy)]-14β,16β-dihydroxy-3-oxobufa-4,20,22-trienolide.

Compound **3** was obtained as an amorphous solid; its molecular formula was determined as $C_{30}H_{42}O_{11}$ from its HR-ESI/TOF-MS (*m/z* 601.2628 $[M+Na]^+$) and ^{13}C NMR spectral data. The IR spectrum of **3** showed absorption bands for the hydroxy groups (3420 cm^{-1}) and carbonyl groups (1734 cm^{-1}). The UV spectrum showed the absorption maxima indicative of a conjugated system (292.2 and 204.2 nm). The ^{1}H and ^{13}C NMR spectra of **3** displayed signals for a 2-pyrone ring moiety characteristic of the bufadienolide skeleton [δ_H 8.14 (dd, J = 9.7, 2.0 Hz, H-22), 7.45 (br d, J = 2.0 Hz, H-21), 6.19 (br d, J = 9.7 Hz, H-23)/δ_C 120.4, 151.8, 152.9, 112.9, and 165.1 (C-20–C-24)]. Furthermore, signals for an aldehyde group [δ_H 9.53 (s, H-19)/δ_C 208.6 (C-19)]; a methyl group [δ_H 0.84 (s, Me-18)/δ_C 17.1 (C-18)]; two hydroxymethine groups [δ_H 4.51 (dd, J = 7.6, 7.6 Hz, H-16)/δ_C 73.4 (C-16), and δ_H 4.12 (br s, H-3)/δ_C 74.3 (C-3)]; and an anomeric proton and carbon group [δ_H 4.35 (d, J = 7.8 Hz, H-1′)/δ_C 102.7 (C-1′)] were observed. Enzymatic hydrolysis of **3** with β-glucosidase gave the aglycone (**3a**) and D-glucose. The above data suggested that **3** was a bufadienolide monoglucoside. The ^{1}H and ^{13}C NMR spectra of **3a** were similar to **1**, except for the signals for rings A and B (C-1–C-10, and C-19). The signals for the C-19 hydroxymethyl group in **1** were displaced by those for an aldehyde group [δ_H 9.55 (s)/δ_C 208.1] in **3a**. Furthermore, the signals assigned to the α,β-unsaturated carbonyl group (C-3, C-4, and C-5) were missing in **3**. Instead, a





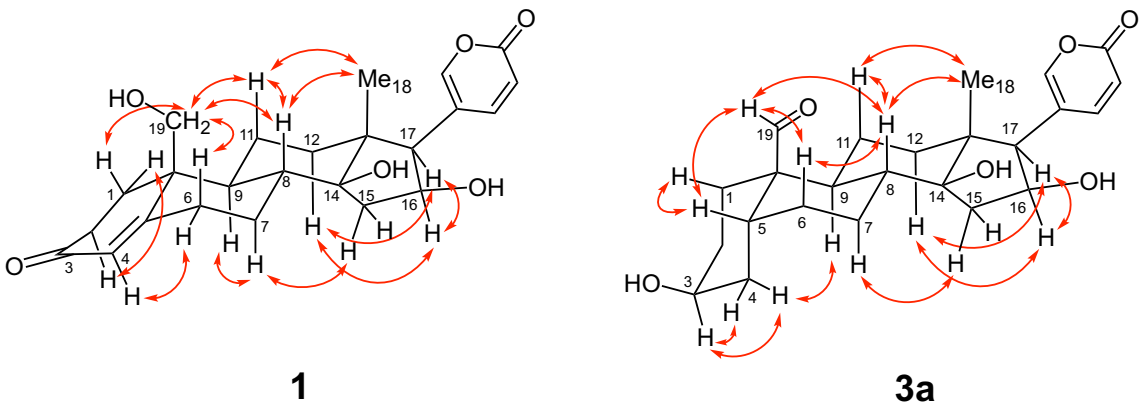


Fig. 2 Key HMBC correlations of **1**–**3**, and **3a**. Bold lines indicate the ^{1}H–^{1}H coupling, and arrows indicate ^{1}H/^{13}C long-range correlations

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

hydroxymethine proton signal at δ_H 4.08 (br s) was observed and was assigned to H-3; the hydroxymethine proton signal showed spin-coupling correlations with adjacent methylene groups at δ_H 1.60 and 1.59 (each m, H_2-2), and δ_H 1.94 and 1.48 (each m, H_2-4) in the ^{1}H–^{1}H COSY spectrum. Therefore, **3a** was a bufadienolide derivative with an aldehyde group at C-19 and three hydroxy groups at C-3, C-14, and C-16. In the PHNOESY spectrum of **3a**, NOE correlations were observed between H-5 and H-1β/H-19, H-8 and H-6β/H-11β/Me-18/H-19, and H-12α and H-16/H-17, indicating that **3a** had the A/B *cis*, B/C *trans*, and C/D *cis* ring fusions, and the 14β-OH, 16β-OH, and 17β-2-pyrone-ring (Fig. 3). Thus, **3a** was determined as 3β,14β,16β-trihydroxy-19-oxo-5β-bufa-20,22-dienolide. The ^{1}H and ^{13}C NMR spectra of **3** showed the presence of a β-D-glucopyranosyl moiety (Glc) [δ_H 4.35 (d, J = 7.8 Hz); δ_C 102.7, 75.2, 78.2,

71.5, 77.9, and 62.8 (C-1′–C-6′)]. In the HMBC spectrum of **3**, long-range correlations were observed between H-1′ of Glc at δ_H 4.35 and C-3 of the aglycone at δ_C 74.3. Accordingly, **3** was assigned as 3β-[(β-D-glucopyranosyl) oxy)]-14β,16β-dihydroxy-19-oxo-5β-bufa-20,22-dienolide.

Compound **4** ($C_{27}H_{44}O_7$) was obtained as an amorphous solid. The IR spectrum of **4** showed absorption bands for hydroxy groups (3417 cm^{-1}) and a carbonyl group (1652 cm^{-1}). The UV spectrum showed the absorption maxima indicative of a conjugated system at 242.1 and 203.2 nm. The ^1H and ^{13}C NMR spectra of **4** showed signals for five methyl groups [δ_H 1.30 (s, Me-21)/δ_C 26.6 (C-21), δ_H 1.19 × 2 (s, Me-26 and Me-27)/δ_C 29.4 (C-27), 29.2 (C-26), δ_H 0.96 (s, Me-19)/δ_C 24.4 (C-19), and δ_H 0.86 (s, Me-18)/δ_C 19.0 (C-18)]; three hydroxymethine groups [δ_H 4.49 (dd, J = 8.3, 6.1 Hz, H-16)/δ_C 72.6 (C-16), δ_H 3.95 (m, H-3)/δ_C 68.7 (C-3), and δ_H 3.84 (m, H-2)/δ_C 68.5 (C-2)]; an olefinic group [δ_H 5.77 (s, H-7)/δ_C 122.2 (C-7)]; and a carbonyl group [δ_C 206.3 (C-6)]. These spectral features of **4** were very similar to those of the known ecdysteroid **5** (taxisterone). The molecular formula of **4** included one more oxygen atom than that of **5**. A comparison of the ^1H and ^{13}C NMR spectra of **4** with those of **5** implied that the C-16 methylene group in **5** was displaced by a hydroxymethine group in **4**. The H-16 hydroxymethine proton at δ_H 4.49 showed spin-coupling correlations with the methylene protons at δ_H 2.42 (dd, J = 14.1, 8.3 Hz, H-15α) and 1.66 (d, J = 14.1 Hz, H-15β) and methine proton at δ_H 2.33 (d, J = 6.1 Hz, H-17) in the ^1H–^1H COSY spectrum of **4**. These data indicated that **4** was 16-hydroxytaxisterone. In the PHNOESY of **4**, an NOE correlation between Me-18 and H-16 allowed the configuration of the C-16 hydroxy group to be determined as α (Fig. 4). Accordingly, **4** was established as (2β,3β,5β,16α)-2,3,14,16,20,25-hexahydroxycholest-7-en-6-one (16α-hydroxytaxisterone).

Four known compounds were identified as taxisterone (**5**) [14], 20-hydroxyecdysone (**6**) [15], stachysterone B (**7**) [16], and shidasterone (**8**) [17], respectively, by comparing their spectroscopic data with those reported previously.

The isolated compounds (**1**–**8**) were evaluated for their cytotoxicity against HL-60, A549, and SBC-3 cells (Table 2). Compounds **1**–**6** were cytotoxic to the three cell lines with IC_{50} values ranging from 0.0055 to 1.9 μM. Among **1**–**8**, compound **3**, a bufadienolide monoglucoside with an aldehyde group at C-19, hydroxy groups at C-14 and C-16, and a (β-D-glucopyranosyl)oxy moiety at C-3, showed the most potent cytotoxic activities against the three cell lines. The new ecdysteroid **4** and taxisterone (**5**) showed almost equal or more potent cytotoxicity compared to the positive controls, cisplatin and etoposide. Compound **6** is the 22-hydroxy derivative of **5** and was less cytotoxic than **5**. Compound **7**, the C-14,15-dehydro derivative (**7**) of **6** and side-chain modification to the five-membered cyclic ether derivative (**8**) of **6**, did not show cytotoxicity at sample concentrations of 20 μM.

Next, the apoptosis-inducing activities of **3** and **4** in HL-60 cells were evaluated. The nuclear chromatin condensation and apoptotic bodies, which are the morphological characteristics of apoptosis cells, were observed in HL-60 cells treated with either **3** or **4** followed by being stained with 4′,6-diamidino-2-phenylindole dihydrochloride (DAPI) (Fig. 5a). In addition, an increase in the sub-G_1 cell population in HL-60 cells treated with either **3** or **4** (**3**: 36.9% of total cells, **4**: 39.1% of total cells) was revealed by cell cycle analysis using a flow cytometer (Fig. 5b). Caspase-3/7, which are the important enzymes that execute apoptosis in apoptotic signaling pathways, were activated when HL-60 cells were treated with either **3** or **4** for 24 h (Fig. 5c). These data suggested that **3** and **4** induced apoptosis in HL-60 cells through the activation of caspase-3/7.

Conclusions

The whole plants of *H. niger* yielded a new bufadienolide (**1**), two new bufadienolide glycosides (**2** and **3**), a new ecdysteroid (**4**), and four known compounds (**5**–**8**). The new bufadienolide glycoside (**2**) has a unique

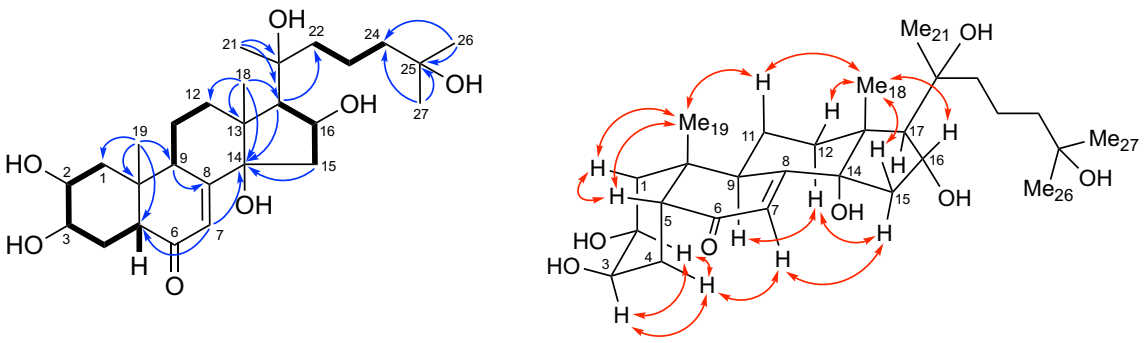
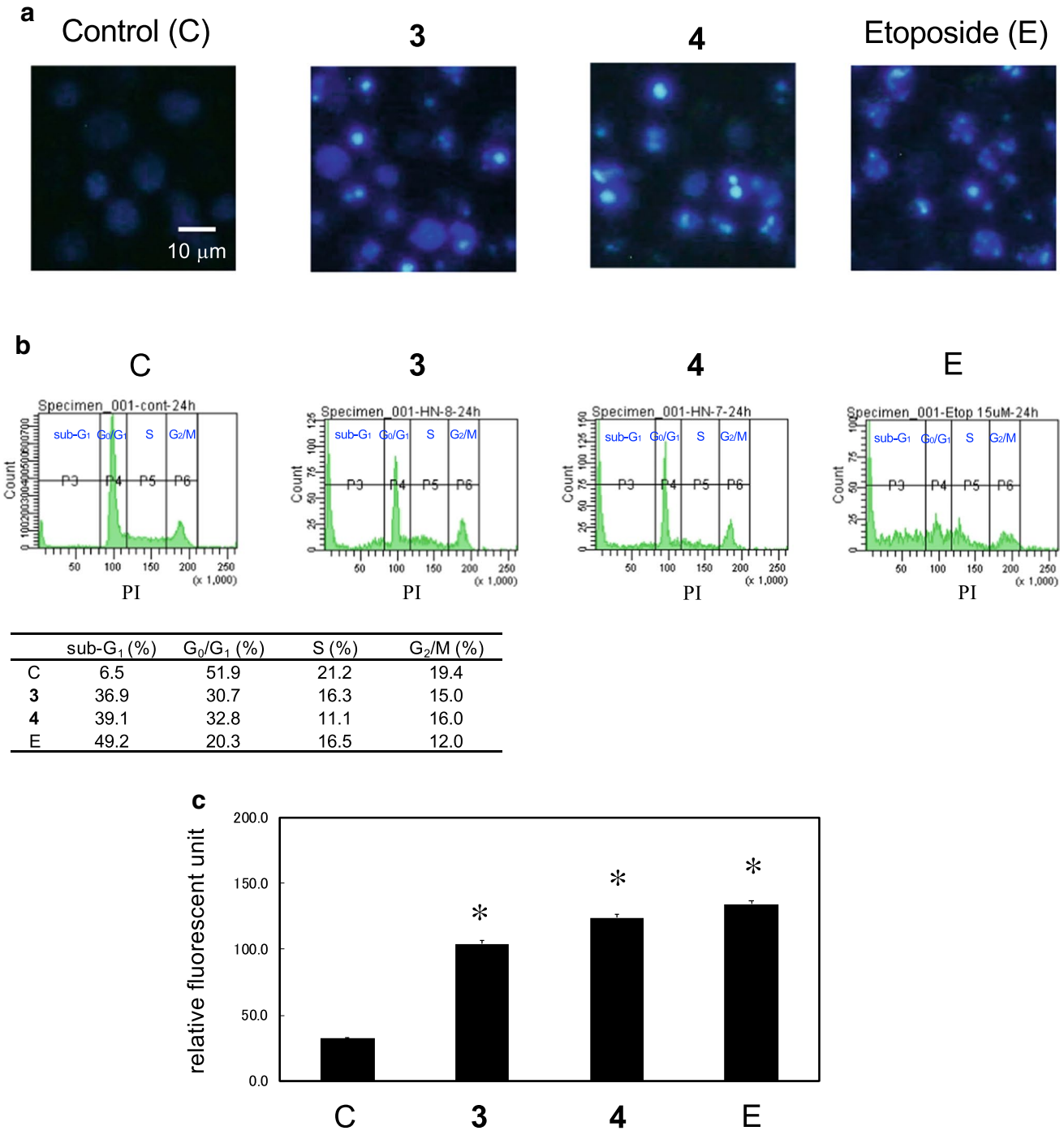


Fig. 4 Key HMBC and NOE correlations of **4**. Bold lines indicate the ^1H–^1H coupling, and arrows indicate $^1H/^{13}C$ long-range correlations



	sub-G_1 (%)	G_0/G_1 (%)	S (%)	G_2/M (%)
C	6.5	51.9	21.2	19.4
3	36.9	30.7	16.3	15.0
4	39.1	32.8	11.1	16.0
E	49.2	20.3	16.5	12.0

Fig. 5 Compounds **3**, **4**, or etoposide induced apoptosis in HL-60 cells. **a** Morphology of HL-60 cells treated with **3**, **4**, or etoposide. HL-60 cells were stained with DAPI after treatment with 0.064 μM of **3**, 2.0 μM of **4**, or 15 μM of etoposide for 24 h. The cells were stained with DAPI and observed by a fluorescence microscopy. **b** Cell cycle progression of the HL-60 cells treated with **3**, **4**, or etoposide. HL-60 cells were treated with 0.064 μM of **3**, 2.0 μM of **4**, or 15 μM of etoposide for 24 h. The cell cycle distribution was analyzed by a flow cytometer. **c** Caspase-3/7 activity in the HL-60 cells treated with **3**, **4**, or etoposide. HL-60 cells were treated with 0.064 μM of **3**, 2.0 μM of **4**, or 15 μM of etoposide for 24 h, and caspase-3 activity was measured using a caspase-3/7 activity apoptosis assay kit. The data are present as mean ± SEM of three experiments. Significantly different from the control group, $^*p < 0.001$

structure in having a D-apiosyl moiety at the C-19 hydroxy group. Compound **6** is a new ecdysteroid derivative (16α-hydroxytaxisterone), and steroids with a 16α-hydroxy group are rarely found in the plant kingdom.

Compounds **1**–**6** showed cytotoxic activities against HL-60, A549, and SBC-3 cells. The new bufadienolide glycoside (**3**) and new ecdysteroid (**4**) induced apoptotic cell death in HL-60 cells via activation of caspase-3/7.

Experimental

General experimental procedures

Optical rotations were measured using a P-1030 (JASCO, Tokyo, Japan) automatic digital polarimeter. IR spectra were recorded on a FT-IR 620 spectrophotometer (JASCO). UV spectra were measured with a V-630 UV–Vis spectrophotometer (JASCO). NMR spectra were recorded on a DRX-600 spectrometer (600 MHz for ^{1}H NMR, Bruker, Karlsruhe, Germany) or a DRX-500 spectrometer (500 MHz for ^{1}H NMR) using standard Bruker pulse programs. Chemical shifts are given as δ values with reference to tetramethylsilane (TMS) as an internal standard. HRESI-TOFMS was recorded on an LCT mass spectrometer (Waters-Micromass, Manchester, UK). Porous-polymer polystyrene resin Diaion HP-20 (Mitsubishi-Chemical, Tokyo, Japan), silica gel Chromatorex BW-300 (300 mesh, Fuji-Silysia Chemical, Aichi, Japan), and ODS silica gel COSMOSIL 75C_{18}-OPN (75 μM particle size, Nacalai Tesque, Kyoto, Japan) were used for CC. TLC was carried out on precoated silica gel 60 F_{254} or RP_{18} F_{254S} plates (0.25 mm thick, Merck, Darmstadt, Germany), and spots were visualized by spraying the plates with 10% H_2SO_4 aqueous solution, followed by heating. HPLC was performed with a system consisting of a LC-20AD pump (Shimadzu, Kyoto, Japan), a Shodex OR-2 detector (Showa-Denko, Tokyo, Japan), and a Rheodyne™ injection port (Thermo Fisher Scientific, Waltham, MA, USA). HL-60 cells (JCRB0085), A549 cells (JCRB0076), and SBC-3 cells (JCRB0818) were obtained from the Japanese Collection of Research Bioresources (JCRB) cell bank (Osaka, Japan). The following materials and reagents were used for the cell culture assays: SH-1300 microplate reader (Corona Electric, Ibaraki, Japan); 96-well flat-bottom plates (Iwaki Glass, Chiba, Japan); fetal bovine serum (FBS), 0.25% trypsin–EDTA solution, RPMI 1640 medium, Dulbecco's Modified Eagle's Medium (DMEM), etoposide, cisplatin, and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2*H*-tetrazolium bromide (MTT), naringinase (EC 3.2.1.40) (Sigma-Aldrich, MO, USA); penicillin G sodium salt, streptomycin sulfate, and phosphate buffered saline (PBS) (Wako Pure Chemical Industries, Osaka, Japan). All other chemicals used were of biochemical reagent grade.

Plant material

The whole plants of *Helleborus niger* were purchased from Hasshinryokusan Co., Ltd (Ibaraki, Japan) in May 2017. A voucher specimen has been deposited in our laboratory (voucher no. KS-2017-015, Department of Medicinal Pharmacognosy).

Extraction and isolation

The whole plants of *H. niger* (4.5 kg) was extracted with hot MeOH (20 L) twice. After removal of the solvent under reduced pressure, the MeOH extract (355 g) was passed through a Diaion HP-20 column successively eluted with H_2O–MeOH (7:3), H_2O–MeOH (1:1), MeOH, EtOH, and EtOAc. The fraction eluted with 50% MeOH exhibited cytotoxicity against HL-60 cells with an IC_{50} value of 0.37 μg/mL. The 50% MeOH eluate (15 g) was subjected to silica gel CC and eluted with stepwise gradient mixtures of EtOAc–MeOH–H_2O (90:10:1; 20:10:1) to produce 9 fractions (A–I). Fraction A was purified by ODS silica gel CC eluted with CH_3CN–H_2O (1:4; 1:2) and MeOH–H_2O (1:3), and by silica gel CC eluted with $CHCl_3$–MeOH–H_2O (190:10:1) to give **8** (5.5 mg). Fraction C was purified by ODS silica gel CC eluted with CH_3CN–H_2O (1:4) and MeOH–H_2O (2:3; 1:1; 3:2) to give **5** (1.6 mg), **6** (309 mg), and **7** (7.3 mg). Fraction D was purified by silica gel CC eluted with $CHCl_3$–MeOH–H_2O (90:10:1; 40:10:1), and by ODS silica gel CC eluted with CH_3CN–H_2O (1:4) and MeOH–H_2O (2:3; 1:1; 3:2) to give **1** (1.0 mg) and **2** (24.7 mg). Fraction E was purified by ODS silica gel CC eluted with CH_3CN–H_2O (1:4) and MeOH–H_2O (3:7), and by silica gel CC eluted with $CHCl_3$–MeOH–H_2O (60:10:1; 40:10:1, 20:10:1) to give **3** (3.5 mg) and **4** (4.7 mg).

14β,16β,19-Trihydroxy-3-oxobufa-4,20,22-trienolide (1)

An amorphous solid; $[\alpha]_D^{25}$-50.3 (*c* 0.031, MeOH); UV (MeOH) λ_{max} nm (log ε): 293.5 (4.02), 241.4 (4.43), 205.5 (4.29); IR (film) ν_{max}: 3358 (OH), 2922 (CH), 1712 and 1650 (C=O) cm^{-1}; HRESI-TOFMS *m/z* 437.1937 $[M+Na]^+$ (calcd for $C_{24}H_{30}NaO_6$, 437.1940); ^{1}H NMR (600 MHz, CD_3OD) and ^{13}C NMR (150 MHz, CD_3OD), see Table 1.

19-[(β-D-Apiofuranosyl)oxy]-14β,16β-dihydroxy-3-oxobufa-4,20,22-trienolide (2)

An amorphous solid; $[\alpha]_D^{25}$ + 42.2 (*c* 0.078, MeOH); UV (MeOH) λ_{max} nm (log ε): 294.2 (3.86), 239.7 (4.27), 205.2 (4.15); IR (film) ν_{max}: 3399 (OH), 2925 (CH), 1707 and 1652 (C=O) cm^{-1}; HRESI-TOFMS *m/z* 569.2369 $[M+Na]^+$ (calcd for $C_{29}H_{38}NaO_{10}$, 569.2363); ^1H NMR (600 MHz, CD_3OD) and ^{13}C NMR (150 MHz, CD_3OD), see Table 1.

3β-[(β-D-Glucopysranosyl)oxy]-14β,16β-dihydroxy-19-oxo-5β-bufa-20,22-dienolide (3)

An amorphous solid; $[\alpha]_D^{25}$ + 32.0 (*c* 0.04, MeOH); UV (MeOH) λ_{max} nm (log ε): 292.2 (3.79), 204.2 (4.19); IR ν_{max} (film) cm^{-1}: 3420 (OH), 2925 (CH), 1734 (C=O); HRESI-TOFMS *m/z* 601.2628 $[M+Na]^+$ (calcd for $C_{30}H_{42}NaO_{11}$, 601.2625); ^1H NMR (600 MHz, CD_3OD) and ^{13}C NMR (150 MHz, CD_3OD), see Table 1.

(2β,3β,5β,16α)-2,3,14,16,20,25-Hexahydroxycholest-7-en-6-one (4)

An amorphous solid; $[\alpha]_D^{25}$ + 32.6 (*c* 0.10, MeOH); UV (MeOH) λ_{max} nm (log ε): 242.1 (4.24), 203.2 (4.09); IR ν_{max} (film) cm^{-1}: 3417 (OH), 2924 (CH), 1652 (C=O); HRESI-TOFMS *m/z* 503.2985 $[M+Na]^+$ (calcd for $C_{27}H_{44}NaO_7$, 503.2985); ^1H NMR (500 MHz, CD_3OD) and ^{13}C NMR (125 MHz, CD_3OD), see Table 1.

Acid hydrolysis of 2

Compounds **2** (2.0 mg) was dissolved in 0.5 M HCl (dioxane–H_2O, 1:1, 2 mL). Then, the solution was heated at 95 °C for 1.5 h under an argon atmosphere. After cooling, the reaction mixture was neutralized by passage through an Amberlite IRA-96SB (Organo, Tokyo) column and subjected to a Diaion HP-20 column eluted with MeOH–H_2O (2:3) followed by EtOH–Me_2CO (1:1) to yield the aglycone fraction and sugar fraction (0.5 mg). The aglycone fraction was chromatographed on silica gel eluted with $CHCl_3$–MeOH–H_2O (90:10:1) to give **1** (0.8 mg). The sugar fractions were analyzed by HPLC under the following conditions: column, Capcell Pak NH2 UG80 (4.6 mm i.d. × 250 mm, 5 µm, Shiseido, Tokyo, Japan); solvent, MeCN–H_2O (17:3); detection, optical rotation (OR); and flow rate: 1.0 mL/min. HPLC analysis of the sugar fraction showed the presence of D-apiose (t_R = 7.55 min, positive optical rotation).

Enzymatic hydrolysis of 3

Compound **3** (1.0 mg) was treated with β-glucosidase (EC 3.2.1.21, Sigma-Aldrich, 5.0 mg) in HOAc/KOAc buffer (pH 5.0, 5 mL) at room temperature for 18 h. The reaction mixture was chromatographed on silica gel eluted with $CHCl_3$–MeOH–H_2O (90:10:1; 20:10:1) to yield 3β,14β,16-trihydroxy-19-oxo-5β-bufa-20,22-dienolide (**3a**, 0.4 mg) and the sugar fraction (0.2 mg). HPLC analysis of the sugar fraction under the same conditions as those of **2** showed the presence of D-glucose (t_R = 17.58 min, positive optical rotation).

3β,14β,16β-Trihydroxy-19-oxo-5β-bufa-20,22-dienolide (3a)

An amorphous solid; $[\alpha]_D^{25}$ + 24.9 (*c* 0.08, MeOH); UV (MeOH) λ_{max} nm (log ε): 205.8 (3.34), 284.3 (2.63); IR ν_{max} (film) cm^{-1}: 3409 (OH), 2924 (CH), 1719 (C=O); HRESI-TOFMS *m/z* 439.2098 $[M+Na]^+$ (calcd for $C_{24}H_{32}NaO_6$, 439.2097); ^1H NMR (600 MHz, CD_3OD), see Table 2; ^{13}C NMR (150 MHz, CD_3OD), see Table 1.

Cell culture and assay for cytotoxic activity against HL-60, A549, and SBC-3 cells

HL-60 cells, A549 cells, and SBC-3 cells were cultured in RPMI 1640 medium (HL-60 cells) or MEM (A549 and SBC-3 cells) containing heat-inactivated 10% (v/v) FBS supplemented with L-glutamine, 100 unit/mL penicillin G sodium salt, and 100 µg/mL streptomycin sulfate in a humidified incubator at 37 °C with 5% CO_2 [18].

The cells (HL-60 cells: 4×10^4 cells/mL, A549 cells: 1×10^4 cells/mL, and SBC-3 cells: 2×10^4 cells/mL) were washed and resuspended in the medium, and the cell suspension (HL-60 cells: 196 µL/well, A549 and SBC-3 cells: 100 µL/well) was seeded into 96-well flat-bottom plates (Iwaki Glass, Chiba, Japan). The cells were continuously

Table 2 Cytotoxic activities of **1**–**8**, cisplatin, and etoposide against HL-60, A549, and SBC-3 cells

	IC_{50} (µM)[a]		
	HL-60	A549	SBC-3
1	0.61 ± 0.016	0.45 ± 0.015	0.20 ± 0.017
2	1.9 ± 0.057	1.4 ± 0.014	0.68 ± 0.053
3	0.013 ± 0.00090	0.0055 ± 0.00020	0.0064 ± 0.00050
4	0.40 ± 0.017	0.27 ± 0.0078	0.46 ± 0.021
5	0.32 ± 0.018	0.19 ± 0.014	0.090 ± 0.00085
6	1.7 ± 0.13	1.3 ± 0.076	1.7 ± 0.051
7	> 20	> 20	> 20
8	> 20	> 20	> 20
Cisplatin	1.4 ± 0.023	2.5 ± 0.14	0.58 ± 0.0085
Etoposide	0.82 ± 0.022	–	0.22 ± 0.0087

[a]Data are presented as the mean ± SEM of three experiments performed in triplicate

treated with each compound for 72 h, and cell viability was measured with an MTT reduction assay procedure. Dose–response curves were plotted for **1**–**8**, and the concentrations giving 50% inhibition (IC_{50}) were calculated.

Morphological observation with DAPI staining

HL-60 cells (5×10^5 cells/well) were plated on coverslips in 96-well plates. The cells were treated with 0.064 μM of **3**, 2.0 μM of **4**, or 15 μM of etoposide for 24 h. The cells were fixed with 1% glutaraldehyde for 30 min at room temperature before staining with DAPI (0.5 μg/mL in PBS). They were then observed immediately through CKX41 fluorescence microscope.

Assay for caspase-3/7 activation

HL-60 cells (1×10^6 cells/well) were treated with 0.064 μM of **3**, 2.0 μM of **4**, or 15 μM of etoposide for 24 h. The activity of caspase-3/7 was measured using the Cell Meter™ caspase-3/7 activity apoptosis assay kit (ABD-22795, AAT Bioquest, Inc., Sunnyvale, CA, USA). Fluorescence intensity was measured using a Varioskan flash fluorescence microplate reader (Thermo Fisher Scientific, Waltham, MA, USA) at Ex/Em = 360/475 nm.

Cell cycle analysis

HL-60 cells (1×10^6 cells) were treated with 0.064 μM of **3**, 2.0 μM of **4**, or 15 μM of etoposide for 24 h. The cells were collected and fixed in 70% EtOH overnight at − 20 °C. Then, the cells were washed with PBS in 1 mL of FxCycle™ (PI)/RNase Staining Solution (Thermo Fisher Scientific) for 15 min. The DNA content was measured using a FACSCanto flow cytometer (BD Biosciences, San Jose, CA, USA).

Acknowledgements This work was financially supported in part by the Japan Society for the Promotion of Sciences (JSPS) KAKENHI (Grant number 18K06735).

Compliance with ethical standards

Conflict of interest The authors declare no conflict of interest associated with this manuscript.

References

1. Tsukamoto Y (ed) (1989) The grand dictionary of horticulture, vol 4. Shogakukan, Tokyo, pp 387–389
2. Meng Y, Whiting P, Sik V, Rees HH, Dinan L (2001) Ecdysteroids and bufadienolides from *Helleborus torquatus* (Ranunculaceae). Phytochemistry 57:401–407
3. Cheng W, Tan YF, Tian HY, Gong XW, Chen KL, Jiang RW (2014) Two new bufadienolides from the rhizomes of *Helleborus thibetanus* with inhibitory activities against prostate cancer cells. Nat Prod Res 28:901–908
4. Zhang H, Su Y, Yang F, Zhao Z, Gao X (2014) Two new bufadienolides and one new pregnane from *Helleborus thibetanus*. Phytochem Lett 10:164–167
5. Watanabe K, Mimaki Y, Sakagami H, Sashida Y (2003) Bufadienolide and spirostanol glycosides from the rhizomes of *Helleborus orientalis*. J Nat Prod 66:236–241
6. Yokosuka A, Iguchi T, Kawahata R, Mimaki Y (2018) Cytotoxic bufadienolides from the whole plants of *Helleborus foetidus*. Phytochem Lett 23:94–99
7. Iguchi T, Uchida Y, Takano S, Yokosuka A, Mimaki Y (2020) Novel steroidal glycosides from the whole plants of *Helleborus foetidus*. Chem Pharm Bull 68:273–287
8. Iguchi T, Yokosuka A, Kawahata R, Andou M, Mimaki Y (2020) Bufadienolides from the whole plants of *Helleborus foetidus* and their cytotoxicity. Phytochemistry 172:112277
9. Iguchi T, Yokosuka A, Tamura N, Takano S, Mimaki Y (2020) Bufadienolide glycosides and bufadienolides from the whole plants of *Helleborus lividus*, and their cytotoxic activity. Phytochemistry 176:112415
10. Duke JA (2002) Handbook of medicinal herbs, 2nd edn. CRC Press LLC, Boca Raton, p 193
11. Vitalini S, Braca A, Fico G (2011) Study on secondary metabolite content of *Helleborus niger* L. leaves. Fitoterapia 82:152–154
12. Duckstein SM, Stintzing FC (2015) LC-MS^n characterization of steroidal saponins in *Helleborus niger* L. roots and their conversion products during fermentation. Steroids 93:47–59
13. Nakamura S, Zhang Y, Nakashima S, Oda Y, Wang T, Yoshikawa M, Matsuda H (2016) Structures of aromatic glycosides from the seeds of *Cassia auriculata*. Chem Pharm Bull 64:970–974
14. Odinokov VN, Galyautdinov IV, Nedopekin DV, Khalilov LM, Shashkov AS, Kachala VV, Dinan L, Lafont R (2002) Phytoecdysteroids from the juice of *Serratula coronata* L. (Asteraceae). Insect Biochem Mol Biol 32:161–165
15. Bandara BMR, Jayasinger L, Karunaratne V, Wannigama GP, Bokel M, Kraus W, Sotheeswaran S (1989) Ecdysterone from stem of *Diploclisia glaucescens*. Phytochemistry 28:1073–1075
16. Simon A, Toth G, Liktor-busa E, Kele Z, Takacs M, Gergely A, Bathori M (2007) Three new steroids from the roots of *Serratula wolffii*. Steroids 72:751–755
17. Roussel PG, Turner NJ, Dinan LN (1995) Synthesis of shidasterone and the unambiguous determination of its configuration at C-22. J Chem Soc Chem Commun 9:933–934
18. Yokosuka A, Sano T, Hashimoto K, Sakagami H, Mimaki Y (2009) Triterpene glycosides from the whole plant of *Anemone hupehensis* var. *japonica* and their cytotoxic activity. Chem Pharm Bull 57:1425–1430