Isolation and Structure of Five New Cancer Cell Growth Inhibitory Bufadienolides from the Chinese Traditional Drug Ch'an Su

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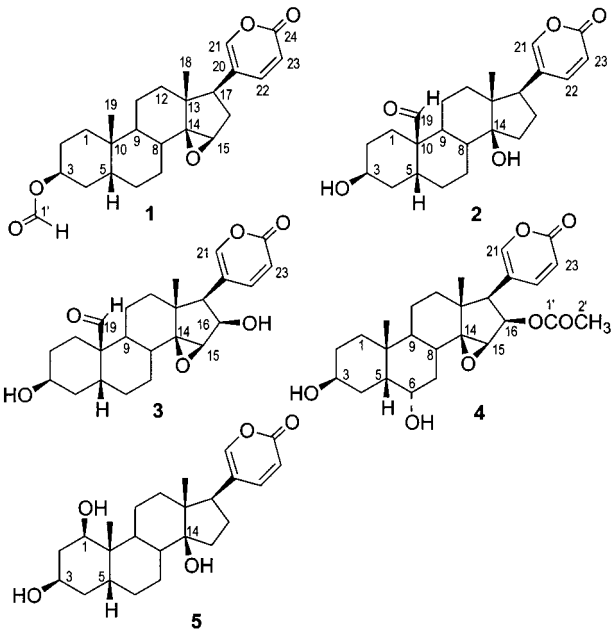
Five new bufadienolides, 3β-formyloxyresibufogenin (**1**), 19-oxobufalin (**2**), 19-oxodesacetylcinobufagin (**3**), 6α-hydroxycinobufagin (**4**), and 1β-hydroxybufalin (**5**), have been isolated together with the previously known bufadienolides **6**−**20** from the Chinese traditional drug "Ch'an Su". The structures were elucidated employing spectroscopic methods. Bufadienolides **1**−**5** provided significant inhibitory activity against the KB and HL-60 cancer cell lines. In addition, bufadienolide **1** was found active against the MH-60 cancer cell line.

The Chinese traditional drug Ch'an Su is a product of the skin secretions of local toads such as *Bufo gargarizans* Cantor or *Bufo melanostrictus* Schneider. The principal biologically active components of Ch'an Su are bufadienolides, which have steroidal A/B *cis* and C/D *cis* ring junctures with a 17β-2-pyrone ring and show a range of activities, such as cardiotonic, blood pressure stimulating, and antineoplastic.[1] Recently, we reported cancer cell growth SAR relationships for a selection of bufadienolides,[2] new separation methods using Sephadex LH-20,[3] and conformational preference of the 17β-2-pyrone ring as analyzed by spectroscopic and computational method.[4] In addition, we have described new indole alkaloid and bufadienolides components of Ch'an Su.[5] In our continuing investigation of Ch'an Su components, we have isolated five new cancer cell growth inhibitory bufadienolides, namely,

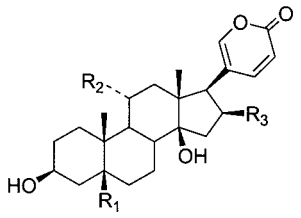


1 **2** **3** **4** **5**

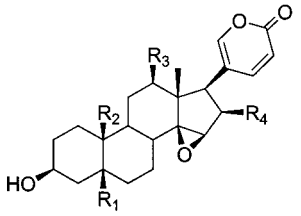
resibufogenin 3-formate (**1**), 19-oxo-bufalin (**2**), 19-oxodesacetylcinobufagin (**3**), 6α-hydroxycinobufagin (**4**), and 1β-hydroxybufalin (**5**). Herein we have summarized the isolation and structure elucidation of bufadienolides **1**−**5** and evaluated their activity along with that of bufadienolides **6**−**20** against three cancer cell lines (KB, HL-60, and MH-60).

Results and Discussion

Thin plate Ch'an Su (2.0 kg) was extracted with CH_3OH and 1:1 C_2H_5OH, and the crude extract was subjected to column chromatography on HP-20 (DIAION). Elution with CH_3OH−water followed by successive column chro-



6, R_1 = OH, R_2 = H, R_3 = $OCOCH_3$
16, R_1 = R_2 = R_3 = H
17, R_1 = R_2 = H, R_3 = $OCOCH_3$
18, R_1 = R_2 = H, R_3 = OH
19, R_1 = OH, R_2 = R_3 = H
20, R_1 = R_3 = H, R_2 = OH



7, R_1 = R_3 = R_4 = H, R_2 = CH_2OH
8, R_1 = R_4 = H, R_2 = CH_3, R_3 = OH
9, R_1 = H, R_2 = CH_3, R_3 = OH, R_4 = $OCOCH_3$
10, R_1 = R_3 = H, R_2 = CH_2OH, R_4 = OH
11, R_1 = R_3 = R_4 = H, R_2 = CH_3
12, R_1 = R_3 = H, R_2 = CH_3, R_4 = $OCOCH_3$
13, R_1 = R_3 = H, R_2 = CH_3, R_4 = OH
14, R_1 = OH, R_2 = CH_3, R_3, = H, R_4 = $OCOCH_3$
15, R_1 = R_4 = OH, R_2 = CH_3, R_3, = H

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Table 1. ^{1}H NMR Spectral Data [500 MHz, δ_H (J, Hz)] for Bufadienolides **1**−**5** in $CDCl_3$ (**1**, **2**, and **5**), CD_3OD (**3**), and $CDCl_3/CD_3OD$ (7:1) (**4**)

position	**1**	**2**	**3**	**4**	**5**
1α	1.60m	1.54m	1.53m	1.54m	3.84s
1β	1.41m	1.81m	1.85m	1.48m	
2α	1.59m	1.54m	1.63(2H)	1.55(2H)	1.76m
2β	1.68m	1.71m			2.00m
3α	5.25br s	4.19br t(2.3)	4.08br t(2.3)	4.15s	4.20m
4α	1.90m	1.87qd(14.2,2.8)	1.95td(13.8,2.8)	1.73m	1.96m
4β	1.48m	1.53m	1.48m	1.51m	1.48m
5β	1.72m	2.33m	2.30m	1.96dt(13.3,4.1)	2.10m
6α	1.23m	1.44m	1.36m		l.39m
6β	1.85dt(13.8,4.6)	1.60m	1.54m	4.09dt(11.9,4.6)	1.85m
7α	0.94qd(12.8,3.7)	1.27m	1.05qd(12.8,3.2)	0.95m	1.32m
7β	1.53m	1.81m	1.48m	1.71m	1.76m
8β	2.01td(11.9,3.7)	1.83m	2.28m	2.12td(12.4,3.7)	1.60td(ll.9,3.7)
9α	1.56m	1.69m	1.82m	1.59m	1.47m
11α	1.53m	1.47m	1.59m	1.56m	1.32m
11β	1.33m	1.81m	1.91td(12.8,3.7)	1.28qd(13.3,3.2)	1.24td(13.7,3.7)
12α	1.40m	1.35td(13.3,3.7)	1.46m	1.44td(13.3,3.2)	1.29m
12β	1.66m	1.55m	1.77m	1.80dt(13.3,2.8)	1.51m
15α	3.53s	2.05dt(12.8,9.6)	3.59s	3.68s	2.00m
15β		1.67m			1.76m
16α	2.39ddd(15.1,10.5,1.4)	2.20dt(12.8,9.6)	4.72dd(9.2,1.4)	5.47d(9.2)	2.l9m
16β	1.96d(15.1)	1.76m			1.71m
17α	2.47d(10.1)	2.47dd(9.6,6.4)	2.69d(9.2)	2.85d(9.2)	2.46dd(9.6,6.4)
18	0.78s	0.78s	0.85s	0.82s	0.71s
19	1.01s	9.50d(1.4)	9.47d(1.8)	0.97s	1.10s
21	7.24d(1.8)	7.24d(2.3)	7.40s	7.22br s	7.23d(l.8)
22	7.79dd(9.6,2.3)	7.84dd(9.6,2.3)	8.10d(9.6)	7.96br s	7.84dd(9.6,2.3)
23	6.25dd(9.6,0.9)	6.27d(9.6)	6.20dd(9.6,0.9)	6.24d(10.2)	6.23d(9.6)
1′	8.07s				
2′				1.91s	

matography on SiO_2, Sephadex LH-20, and C_{18}−HPLC afforded new compounds **1**−**5** as colorless solids together with the previously known bufadienolides, resibufogenin 3-formate (**1**, 11.2 mg, 0.0012%), 19-oxobufalin (**2**, 25.1 mg, 0.0028%), 19-oxodesacetylcinobufagin (**3**, 30.3 mg, 0.0034%), 6α-hydroxycinobufagin (**4**, 12.1 mg, 0.0013%), 5β-hydroxybufotalin (**5**, 10.8 mg, 0.0012%), and 1β-hydroxybufalin (**6**, 52.2 mg, 0.0058%) as colorless solids together with the previously known bufadienolides, 5β-hydroxybufotalin (**6**), 19-hydroxyresibufogenin (= resibufaginol) (**7**), 12β-hydroxyresibufogenin (**8**), 12β-hydroxycinobufagin (**9**), 19-hydroxydesacetylcinobufagin (= desacetylcinobufaginol) (**10**), resibufogenin (**11**), cinobufagin (**12**), desacetylcinobufagin (**13**), cinobufotalin (**14**), desacetylcinobufotalin (**15**), bufalin (**16**), bufotalin (**17**), desacetylbufotalin (**18**), telocinobufagin (**19**), and gamabufotalin (**20**). Structures **1**−**5** were assigned as follows.

Bufadienolide **1** gave a pseudomolecular ion at m/z 413 $[M + H]^+$ in the FABMS corresponding to the molecular formula $C_{25}H_{32}O_5$. The UV and IR absorption spectra implied the presence of 2-pyrone (298 nm; 1653 cm^{-1}) and ketone (1717 cm^{-1}) groups. Analysis of ^{1}H and ^{13}C NMR data (Tables 1 and 2) and the HMQC spectrum confirmed that steroid **1** possessed 25 carbon atoms. Among these, three sp^2 carbons (δ_c 149.59, 162.02, and 160.73), two sp^3 methines (δ_c 59.85 and 70.47), and one sp^3 quaternary carbon (δ_c 74.60) were assigned to carbon atoms bearing an oxygen atom. The ^{1}H NMR spectrum was similar to that of resibufogenin (**11**) with H-21, H-22, and H-23 signals (δ_H 7.24, 7.79, and 6.25) characteristic of the 2-pyrone ring of bufadienolides and H-15 (δ_H 3.53) of a 14,15-epoxy group. The ^{1}H−^{1}H COSY spectrum revealed the connectivities of C-2 to C-5, C-6 to C-9, and C-22 to C-23. In the HMBC spectrum, correlations of H-3 to C-1 and C-5, H-12 to C-9, C-11, and C-14, H-17 to C-12, C-14, and C-15, and H-16 to C-13 and C-14 were observed. These results showed the connectivities of C-1 to C-5 and C-6 to C-17. In addition, HMBC correlations of H-19 to C-1, C-5, and C-9 and H-18 to C-12, C-14, and C-17 suggested that the two methyl groups (C-19 and C-18) were located on C-10 (δ_c 35.28) and C-13 (δ_c 45.27), respectively. The formyl signal correlated to C-3 in the HMBC spectrum and was assigned as H-1′. The connectivity between C-17 and C-20 was supported by HMBC correlations of H-17 to C-21 and C-22 and H-16 to C-20, thus giving rise to the connectivity through C-20 of the 2-pyrone ring.

Table 2. ^{13}C NMR Data (125 MH_z δC) of Bufadienolides **1**−**5** (cf. Table 1 Solvents)

position	**1**	**2**	**3**	**4**	**5**
1	30.20	21.09	21.79	29.83	73.48
2	25.07	26.38	26.91	27.34	32.40
3	70.47	65.56	66.47	65.57	68.24
4	30.42	32.33	33.03	25.95	33.51
5	36.73	28.80	30.13	42.48	30.11
6	25.60	28.07	28.61	66.75	26.01
7	20.66	21.37	21.98	28.31	21.43
8	33.59	42.50	34.78	32.39	42.44
9	39.55	34.90	38.96	38.78	37.60
10	35.28	51.06	52.45	36.76	39.95
11	21.07	21.00	21.79	20.96	21.02
12	39.34	40.86	40.80	40.07	40.79
13	45.27	48.46	46.35	45.36	48.23
14	74.60	85.04	73.23	72.15	85.18
15	59.85	31.87	63.23	59.61	32.75
16	32.42	28.56	73.13	74.93	28.70
17	47.80	51.11	53.15	50.40	51.23
18	16.86	16.42	17.57	17.33	16.56
19	23.70	205.97	207.56	23.88	18.83
20	122.22	122.59	119.86	116.64	122.60
21	149.59	148.63	152.71	151.64	148.60
22	146.96	146.72	151.97	148.96	146.73
23	115.32	115.37	113.64	113.38	115.37
24	162.02	162.36	164.82	162.51	162.39
1′	160.73			170.57	
2′				20.53	

The phase-sensitive NOESY spectrum was useful for analysis of the stereochemistry of bufadienolide **1**. NOESY correlations of H_3-19/H-5, H_3-19/H_b-6, H_3-19/H_b-8, and H_3-

Table 3. Cancer Cell Growth Inhibitory Activity of the New Bufadienolides (**1**−**5**) and Related Bufadienolides (**6**−**20**)

	cancer cell lines IC_{50} (μg/mL)								
compd	Kb[a]	HL60[b]	MH60[c]	BXPC3[d]	MCF7[e]	SF268[f]	NCIH460[g]	KM20L2[h]	DU145[i]
1	3.40	1.00	8.1	1.6	0.60	0.38	0.53	0.54	0.42
2		<0.01	>25	0.014	0.0072	0.0047	0.018	0.0082	0.0046
3	0.65	3.00	>25	>1	>1	>1	>1	>1	>1
4	0.87	0.038	>25	0.46	0.360	0.32	0.74	0.28	0.21
5	0.19	<0.01	>25	0.024	0.012	0.0044	0.014	0.011	0.005
6	0.20	<0.01	>25	0.11	0.046	0.033	0.048	0.034	0.024
7	1.20	0.48	>25	0.63	0.33	0.25	0.44	0.45	0.38
8	0.97	0.045	>25	0.12	0.066	0.046	0.017	0.012	0.041
9	0.79	<0.01	>25						
10	3.90	0.49	>25						
11	1.30	0.50	10						
12	0.21	<0.01	>25						
13	0.44	1.00	>25						
14	0.37	0.047	>25						
15	10.00	4.30	>25						
16	0.67	<0.01	>25						
17	0.19	<0.01	>25						
18	0.79	0.025	>25						
19	1.30	<0.01	>25						
20	0.75	0.014	>25						

[a] Human carcinoma of the nasopharyx. [b] Human myelocytic leukemia. [c] Murine leukemia. [d] Pancreas-a. [e] Breast adn. [f] CNS gliobl. [g] Lung-NSC. [h] Colon. [i] Prostate.

19/H_b-11 indicated that those protons were β-oriented. The *cis* ring junction at C-5 and C-10 was deduced from the NOESY correlation of H_a-4/H_a-7 and two six-membered rings had chair conformations, which was suggested by relatively small proton coupling constants between H-5 and H-6. Additional NOESY correlations of H_3-18/H_b-11, H_3-18/H-8, and H_a-12/H_a-17 argued well for the stereochemistry of B/C *trans*, C/D *cis* ring junctures, and C-17. In addition, the β-epoxide at C-14−15 was verified and provided additional proof of chair conformations for steroid rings A−C. Complete assignments for the ^{1}H and ^{13}C NMR spectra were summarized in Tables 1 and 2. Thus, the structure of **1** was assigned as resibufogenin 3-formate (**1**).

The molecular formulas of compounds **2** and **3** were determined to be $C_{24}H_{32}O_5$ and $C_{24}H_{30}O_6$, respectively, by HRFABMS. Again the UV and IR absorption spectra suggested the presence of a 2-pyrone ring, supported by ^{1}H and ^{13}C NMR data (Tables 1 and 2). The NMR spectra were similar to those of bufalin (**16**) and desacetylcinobufagin (**13**), respectively. However, the 19-methyl signals were missing and new signals were observed at δ_H 9.50 and δ_C 205.97 for **2** and δ_H 9.47 and δ_C 207.56 for **3**, implying 19-oxobufadienolide structures. In the HMBC spectra of steroids **2** and **3**, the H-19 signals assignable to the aldehyde showed an HMBC correlation to C-9. Furthermore, HMBC correlations of H-9 to C-19 were also observed. In addition, the remaining NMR data was consistent with that of bufadienolides **16** and **13**, respectively. That correlation allowed assignment of steroid **2** as 19-oxobufalin and steroid **3** as 19-oxodesacetylcinobufagin. In the ^{1}H NMR spectra of bufadienolides **2** and **3**, the 19-aldehyde signals were observed as doublets arising from coupling with the H-5 signals.

Compound **4** corresponded to molecular formula $C_{26}H_{34}O_7$ by HRFABMS. The UV and IR absorption spectra again suggested the presence of a 2-pyrone ring, and this was confirmed by the ^{1}H and ^{13}C NMR data (Tables 1 and 2). In the ^{1}H NMR spectrum, the H-21 and H-22 signals were broad singlets. The ^{1}H NMR signals for H-15 (δ_H 3.68) of the 14,15-epoxy group and H-16 (δ_H 5.47) of the 16-acetoxyl group (appeared at δ_H 1.91) were also similar to those of cinobufagin (**12**). However, a new double doublet signal appeared at δ_H 4.06 for a carbon atom bearing a hydroxyl group. In the ^{13}C NMR spectrum, an oxygenated carbon signal was found at δ_C 66.75. The ^{1}H−^{1}H COSY spectrum revealed connectivities of C-2−C-9, and the new proton signal was assigned as H-6. This was supported by a HMBC correlation of H-6 to C-4. The orientation of H-6 was assigned as β by correlations with axial protons H-8 and H-19 in the NOESY spectrum. Thus, the structure of **4** was determined to be 6α-hydroxycinobufagin.

Bufadienolide **5** was found to have molecular formula $C_{24}H_{34}O_5$ by HRFABMS. The ^{1}H and ^{13}C NMR spectra (Tables 1 and 2) were similar to that of the bufalin (**16**) spectra except for an additional signal at δ_H 3.84, which implied the addition of a new hydroxyl group. Analysis of the HMQC spectrum emphasized the presence of a new oxygenated methine carbon signal at δ_C 73.48, which was assigned to C-1 by an HMBC correlation with H-19 and of H-1 (δ_H 3.84) to C-3 and C-5. In the NOESY spectrum, a correlation between H-1 and H-11 suggested that H-1 was α-oriented and the hydroxyl group was β, thereby completing the structure determination of 1β-hydroxybufalin (**5**).

Among the previously known bufadienolides (**6**−**20**) we isolated from Ch'an Su during this investigation, three (**6**, **7**, and **8**) had not been discovered earlier as natural products. Instead bufadienolides **6**, **7**, and **8** had been obtained by semisynthesis from, respectively, bufotalin (**17**), resibufagin,[6] and resibufogenin (**11**).[7] The structural elucidation of bufadienolides **6**, **7**, and **8** was accomplished employing the analogous spectral interpretation procedures used for assigning structures **1**−**5**.

All (**1**−**20**) of the Ch'an Su bufadienolides were found to be active against the KB and HL-60 cancer cell lines (Table 3). The new bufadienolides (**1**−**5**) were also evaluated against a minipanel of human cancer cell lines (Table 3). Of these, the bufalin derivatives **2** and **5** were found to be exceptionally inhibitory (IC_{50} to 10^{-3} μg/mL). Those were followed closely by 5β-hydroxybufotalin (**6**). Two (**1** and **11**) also inhibited growth of the MH-60 murine leukemia cell line. In our early studies of toad venom, bufadienolides in general were found to inhibit growth of the human carcinoma of the nasopharynx (KB) cell line[8] and more recently were examined in detail (including SAR) against the liver carcinoma PLC/PRF/5 cell line.[2] Usually, the HL-60 cell line is more sensitive to the natural bufadienolides (cf. Table 3). Interestingly, bufalin (**16**) is believed to occur in human serum and may be protective against leukemia.[1,9]

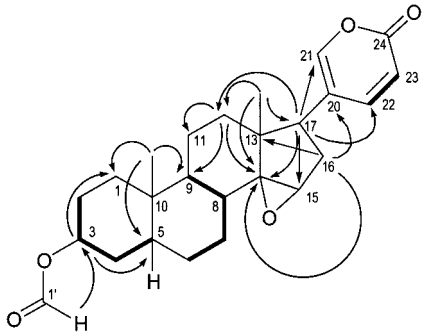

Figure 1. Selected 2D NMR correlations for 3β-formyloxy-resibufogenin (**1**).

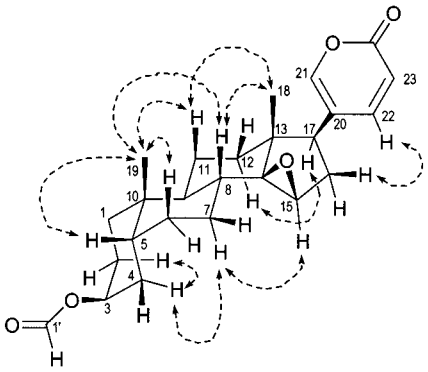

Figure 2. Selected NOESY correlations for 3β-formyloxy-resibufogenin (**1**).

The isolation of new bufadienolides from plants[10] and animal sources[11] continues along with SAR studies and should lead to additional substances with medical potential.

Experimental Section

General Experimental Procedures. Solvents and reagents of analytical grade were purchased from commercial sources. Thin-plate Ch'an Su, which was a black rectangular thin plate (15 cm × 23 cm × 0.1 cm) prepared by Shanghai Medicinal Herbs Import and Export Corp. (Shanghai), was purchased in a Hong Kong folk-medicine market in 1995. The UV spectra, IR spectra, and optical rotations were measured using a SHIMADZU UV mini 1240 UV−vis spectrometer, JASCO FT/IR-300 spectrometer, and HORIBA SEPA-300 high sensitive polarimeter, respectively. FABMS were recorded with a JEOL JMS-AX 505H using *m*-nitrobenzyl alcohol as a matrix (positive mode) or with a JEOL LC-Mate using a glycerol matrix. NMR spectra were recorded at room temperature using a JEOL EX-500 spectrometer with 500 MHz for ^{1}H and 125 MHz for ^{13}C. Compounds were dissolved in $CDCl_3$ (for **1**, **2**, and **5**), CD_3OD (for **3**), or 7:1 $CDCl_3$/CD_3OD (for **4**) owing to solubility behavior and measured using 5 mm sample tubes. Chemical shifts (in ppm) were referenced to tetramethylsilane as internal standard. Coupling constants (*J* values) were expressed in Hz. All 2D NMR experiments were measured using the field gradient mode. NOESY spectra were recorded with 1.0 s mixing time and processed in the phase-sensitive mode. For chromatographic separation, HP-20 (DIAION, Mitsubishi Chemical), Sephadex LH-20 (Pharmacia Biotec), and SiO_2 (Silica gel 60 spherical, KANTO chemical) were used. HPLC was performed with an Inertsil ODS-3 column (20 mm i.d. × 250 mm, GL Science) packed with 5 μm ODS. TLC was conducted on Uniplate silica gel GF_{254} TLC plates from ANALTECH. The spotes were detected by UV light at 254 nm (UV GL-25 Mineralight lamp, Upland, CA) and color reaction spraying with 5% H_2SO_4−ethanol reagent and heating at 140 °C.

Extraction and Isolation. Thin-plate Ch'an Su (2.0 kg) was ground into a rough powder and extracted successively (3×) by methanol and 1:1 ethanol−water for 5 days at room temperature. The extract was concentrated (reduced pressure) to provide 600 g of residue. Part of the residue (270 g) was subjected to column chromatography on HP-20, eluting with a gradient of CH_3OH−water by increasing the CH_3OH (20−100%), to afford five fractions. Fraction 5 was rich in bufadienolides by TLC (developed by *n*-hexane−CH_2Cl_2−acetone (4:3:3) inspection and subjected to column chromatography on Sephadex LH-20 (eluting with 1:1 CH_2Cl_2−CH_3OH). The bufadienolide fraction was rechromatographed on Sephadex LH-20 eluting with *n*-hexane−CH_2Cl_2−CH_3OH (4:5:1). Column chromatography on SiO_2, Sephadex LH-20, and HPLC was used repeatedly for separation and followed by checking the color reactions on TLC. By these methods, resibufogenin 3-formate (**1**, 11.2 mg, 0.0012%), 19-oxobufalin (**2**, 25.1 mg, 0.0028%), 19-oxodesacetylcinobufagin (**3**, 30.3 mg, 0.0034%), 6α-hydroxycinobufagin (**4**, 12.1 mg, 0.0013%), 5β-hydroxybufalin (**5**, 52.2 mg, 0.0058%), 5β-hydroxybufotalin (**6**, 10.8 mg, 0.0012%), 19-hydroxyresibufogenin (= resibufaginol) (**7**, 71.3 mg, 0.0079%), 12β-hydroxyresibufogenin (**8**, 50.6 mg, 0.0056%), and 12β-hydroxycinobufagin (**9**, 9.3 mg, 0.0010%) were obtained. In addition, the 10 well-known bufadienolides, resibufogenin (**11**), cinobufagin (**12**), desacetylcinobufagin (**13**), cinobufotalin (**14**), desacetylciobufotalin (**15**), bufalin (**16**), bufotalin (**17**), desacetylbufotalin (**18**), telocinobufagin (**19**), and gamabufotalin (**20**), were isolated and identified by direct comparison with authentic samples. Bufadienolides were also detected in fraction 4 by TLC. When that fraction was subjected to column chromatography on Sephadex LH-20, SiO_2, and by HPLC as with fraction 5, the result was 19-hydroxydesacetylcinobufagin (= desacetylcinobufaginol) (**10**, 108.0 mg, 0.012%).

Resibufogenin 3-formate (1): colorless solid; $[\alpha]_D^{21}$ +12.0° (*c* 0.1, CH_3OH); UV (CH_3OH) λ_{max} (log ϵ) 298 nm (3.4); IR (KBr) ν_{max} 3447, 2928, 1717, 1653, 1636, 1540, 1456, 1257, 1187, 1155, and 1121 cm^{-1}; FABMS *m*/*z* 413 $(M + H)^+$ and 435 $(M + Na)^+$; HRFABMS *m*/*z* 413.2335 [calcd for $C_{25}H_{33}O_5$ $(M + H)^+$, 413.2328]; ^{1}H and ^{13}C NMR data are recorded in Tables 1 and 2.

19-Oxobufalin (2): colorless solid; $[\alpha]_D^{21}$ +7.0° (*c* 0.1, CH_3OH); UV (CH_3OH) λ_{max} (log ϵ) 299 nm (3.6); IR (KBr) ν_{max} 3416, 2926, 1713, 1633, 1538, 1451, 1129, 1065, 1028, 947, and 833 cm^{-1}; FABMS *m*/*z* 401 $(M + H)^+$ and 423 $(M + Na)^+$; HRFABMS *m*/*z* 401.2358 [calcd for $C_{24}H_{33}O_5$ $(M + H)^+$, 401.2328]; ^{1}H and ^{13}C NMR data appear in Tables 1 and 2.

19-Oxodesacetylcinobufagin (3): colorless solid; $[\alpha]_D^{21}$ +17.3° (*c* 0.1, CH_3OH); UV (CH_3OH) λ_{max} (log ϵ) 295 nm (3.6); IR (KBr) ν_{max} 3379, 2925, 1708, 1536, 1449, 1134, and 1029 cm^{-1}; FABMS *m*/*z* 415 $(M + H)^+$ and 437 $(M + Na)^+$; HRFABMS *m*/*z* 415.2135 [calcd for $C_{24}H_{31}O_6$ $(M + H)^+$, 415.2121]; ^{1}H and ^{13}C NMR data in Tables 1 and 2.

6α-Hydroxycinobufagin (4): colorless solid; $[\alpha]_D^{21}$ −3.2° (*c* 0.1, CH_3OH); UV (CH_3OH) λ_{max} (log ϵ) 294 nm (3.7); IR (KBr) ν_{max} 3417, 2927, 1714, 1633, 1537, 1454, 1374, 1240, 1131, 1040, and 881 cm^{-1}; FABMS *m*/*z* 459 $(M + H)^+$ and 481 $(M + Na)^+$; HRFABMS *m*/*z* 459.2406 [calcd for $C_{26}H_{35}O_7$ $(M + H)^+$, 459.2383]; ^{1}H and ^{13}C NMR data in Tables 1 and 2.

5β-Hydroxybufotalin (5): colorless solid; $[\alpha]_D^{21}$ −18.7° (*c* 0.1, CH_3OH); UV (CH_3OH) λ_{max} (log ϵ) 295 nm (3.6); IR (KBr) ν_{max} 3394, 2926, 1704, 1651, 1635, 1539, 1456, 1249, 1089, 1047, and 834 cm^{-1}; FABMS *m*/*z* 461 $(M + H)^+$; HRFABMS *m*/*z* 461.2485 [calcd for $C_{26}H_{37}O_7$ $(M + H)^+$, 461.2538]; ^{1}H and ^{13}C NMR data are shown in Tables 1 and 2.

Biological Evaluation. Human oral epidemoid carcinoma KB cells, human leukemia HL-60 cells, and murine leukemia MH-60 cells were maintained in culture flasks in MEM with KB, RPMI1640 for HL-60, and MH-60 supplemented with 10% FBS and kanamaycin sulfate (100 μg/mL), respectively. For the in vitro drug treatment experiments, tumor cells (2×10^4 cells for KB and HL-60 cells, 5×10^3 cells for MH-60) were seeded in 0.2 mL of culture medium/well in 96-well plates. The cells were treated in triplicate with graded concentrations of 5 μL test samples and were then incubated in 5% carbon dioxide atmosphere at 37 °C for 72 h. The MTT cytotoxicity assay was used to measure the cytotoxic effect expressed as

IC_{50}, the concentration of test compound (μg/mL) to give 50% inhibition of cell growth.

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References and Notes

(1) (a) Krenn, L.; Kopp, B. *Phytochemistry* **1998**, *48*, 1−29. (b) Numazawa, S.; Honma, Y.; Yamamoto, T.; Yoshida, T.; Kuroiwa, Y. *Leukemia Res.* **1995**, *19*, 945−953. (c) Jing, Y.; Ohizumi, H.; Kawazoe, N.; Hashimoto, S.; Masuda, Y.; Nakajo, S.; Yoshida, T.; Kuroiwa, Y.; Nakaya, K. *Jpn. J. Cancer Res.* **1994**, *54*, 645−651.

(2) Kamano, Y.; Kotake, A.; Hashima, H.; Inoue, M.; Morita, H.; Takeya, K.; Itokawa, H.; Nandachi, N.; Segawa, T.; Yukita, A.; Saitou, K.; Katsuyama, M.; Pettit, G. R. *Bioorg. Med. Chem.* **1998**, *6*, 1103−1115.

(3) Kamano, Y.; Nogawa, T.; Kotake, A.; Tozawa, M.; Pettit, G. R. *J. Liq. Chrom., Relat. Technol.* **1999**, *22*, 2455−2465.

(4) (a) Kamano, Y.; Kotake, A.; Nogawa, T.; Hashima, H.; Tozawa, M.; Morita, H.; Takeya, K.; Itokawa, H.; Matsuo, I.; Ichihara, Y.; Drasar, P.; Pettit, G. R. *Collect. Czech. Commun.* **1998**, *63*, 1663−1670. (b) Kamano, Y.; Kotake, A.; Hashima, H.; Morita, H.; Takeya, K.; Itokawa, H. *Heterocycles* **1998**, *49*, 275−279.

(5) (a) Kamano, Y.; Morita, H.; Takano, R.; Kotake, A.; Nogawa, T.; Hashima, H.; Takeya, K.; Itokawa, H.; Pettit, G. R. *Heterocycles* **1999**, *50*, 499−503. (b) Kamano, Y.; Inoue, M.; Pettit, G. R.; Satoh, H.; Tozawa, M. *Tennen Yuki Kagobutsu Toronkai Koen Yoshishu*, 22nd ed.; Kyushu Daigaku Rigakubu Kagakka: Fukuoka, Japan, 1979; p 33.

(6) Kamano, Y.; Yamamoto, H.; Hatayama, K.; Tanaka, Y.; Shinohara, M.; Komatsu, M. *Tetrahedron Lett.* **1968**, *54*, 5669−5672.

(7) Schüpbach, M.; Tamm, C. *Helv. Chim. Acta* **1969**, *47*, 2217−2226.

(8) Ode, R. H.; Pettit, G. R.; Kamano, Y. In *Steroids*; Hey, D. H., Johns, W. F., Eds.; Buttersworths: Boston, 1976; Vol. 8, p 145; *ibid*. 1973; p 151.

(9) Watabe, M.; Kawazoe, N.; Masuda, Y.; Nakajo, S.; Nakaya, K. *Cancer Res.* **1997**, *57*, 3097−3100.

(10) Krenn, L.; Kopp, B.; Deim, A.; Robien, W.; Kubelka, W. *Planta Med.* **1993**, *60*, 63−69.

(11) Matsukawa, M.; Akizawa, T.; Morris, J. F.; Butler, V. P.; Yoshioka, M. *Chem. Pharm. Bull.* **1996**, *44*, 255−257.

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