Isolation and Structure of a 20,21-Epoxybufenolide Series from "Ch'an Su"

Yoshiaki Kamano,[†] Toshihiko Nogawa,[†] Ayano Yamashita,[†] Masahiko Hayashi,[‡] Masuo Inoue,[§] Pavel Drašar,[⊥] and George R. Pettit*[,§]

Faculty of Science, Kanagawa University, 2946 Tsuchiya, Hiratsuka, Kanagawa 259-1293, Japan, Kitasato Institute, Shirogane, Minato-ku, Tokyo 108-8642, Japan, Cancer Research Institute and Department of Chemistry and Biochemistry, Arizona State University, Tempe, Arizona 85287-2404, and Institute of Organic Chemistry and Biochemistry, Academy of Sciences of the Czech Republic, 166 10 Prague, Czech Republic

Received January 31, 2002

Steroid bufenolides resulting from epoxidation of the 17β-2-pyrone ring of bufadienolides are rare. Five 20,21-epoxybufenolides, namely, 20*S*,21-epoxyresibufogenin (**1**), 20*R*,21-epoxyresibufogenin (**2**), 3-*O*-formyl-20*S*,21-epoxyresibufogenin (**3**), 3-*O*-formyl-20*R*,21-epoxyresibufogenin (**4**), and 3-oxo-20*S*,21-epoxyresibufogenin (**5**), were isolated from the Chinese toad skin extract drug Ch'an Su. The structures were elucidated by spectroscopic and chemical methods. The configuration at C-20 was assigned by the analysis of difference NOE spectra. The cancer cell (KB and MH-60) growth inhibition by the new 20,21-epoxybufenolides was examined, and 20,21-epoxides **1**, **2**, and **5** were found to significantly inhibit the leukemia MH-60 cell line.

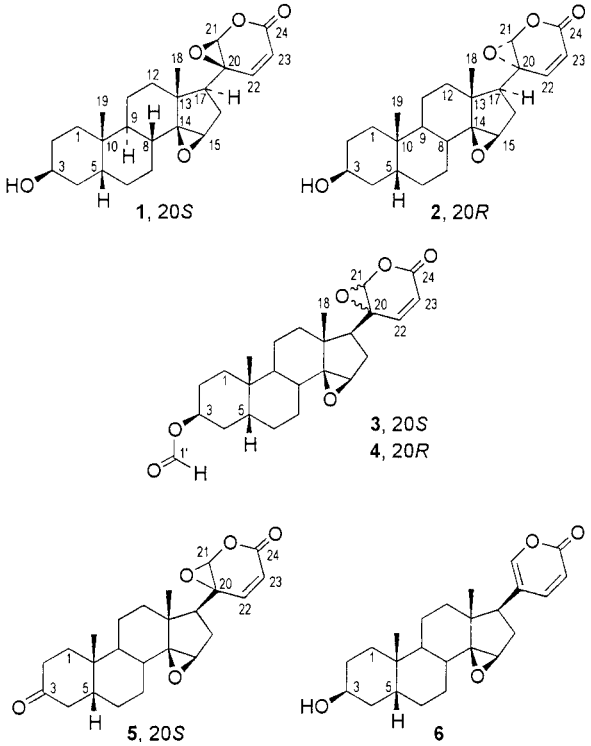
The isolation and structural determination of interesting new and potentially useful steroids of the bufadienolide class from plants[1] and animals[2] continues to advance productively. In the early phases of our investigation[3] of bufadienolides and potential cancer cell growth inhibitors, the Chinese traditional drug Ch'an Su[4] prepared from skin secretions of local toads[2a] proved to be a useful source for a variety of bufadienolides and new members as our separation techniques advanced.[5] Recently we completed the isolation and structural elucidation of five new and 15 previously known bufadienolides from Ch'an Su.[2a]

Bufadienolides bearing epoxide substitution in the steroid nucleus especially at C-14 and C-15[2a,3,6] are well known and include a new nuclear example: 11,19-epoxy-19-methoxytelocinobufagin (marinosin).[2b] We herein report three new Ch'an Su bufenolide constituents and two (**1** and **2**) that were earlier described[6,7] (but without side-chain stereochemical assignments) bearing the rarely[7,8] encountered 17β-2-pyrone ring epoxide group, namely, 20*S*,21-epoxyresibufogenin (**1**), 20*R*,21-epoxyresibufogenin (**2**), 3-*O*-formyl-20*S*,21-epoxyresibufogenin (**3**), 3-*O*-formyl-20*R*,21-epoxyresibufogenin (**4**), and 3-oxo-20*S*,21-epoxyresibufogenin (**5**).

The existence of 20,21-epoxyresibufogenin had been earlier (1965) suspected from TLC of analyses. The isolation and structure elucidation was subsequently reported.[8] However, the stereochemistry of the 20,21-epoxy group was not determined. Therefore, we examined the difference NOE spectra and determined the relative configuration at C-20 for each of the 20,21-epoxides isolated from Ch'an Su. In addition, we synthesized authentic specimens of the 20,21-epoxybufenolides.

Results and Discussion

Ch'an Su, obtained in Hong Kong, was extracted with CH_2Cl_2, and the crude extract was subjected to column chromatography on SiO_2 eluting with *n*-hexane−acetone,



followed by column chromatography on SiO_2 and Sephadex LH-20 to afford 20*S*,21-epoxyresibufogenin (**1**, 0.22%), 20*R*,21-epoxyresibufogenin (**2**, 0.16%), 20*S*,21-epoxyresibufogenin 3-formate (**3**, 0.0022%), 20*R*,21-epoxyresibufogenin 3-formate (**4**, 0.00082%), and 3-oxo-20*S*,21-epoxyresibufogenin (**5**, 0.0048%) together with the known resibufogenin (**6**).

Pyrone epoxide **1** was obtained as colorless plates, and the molecular formula $C_{24}H_{32}O_5$ was established by HRFABMS [*m*/*z* 401.2252 $(M + H)^+$, Δ −7.6 mmu]. The UV spectrum showed maximum absorption at 232 nm, which indicated a profound change in the α-pyrone ring. That was supported by the IR spectrum, which showed that the characteristic absorption of the lactone carbonyl at 1720 cm^{-1} was shifted to 1744 cm^{-1}. Analysis of ^1H and ^{13}C NMR data (Tables 1 and 2) and the HMQC spectrum provided

* To whom correspondence should be addressed. Tel: (480) 965-3351. Fax: (480) 965-8558.
[†] Kanagawa University.
[‡] Kitasato Institute.
[§] Cancer Research Institute, Arizona State University.
[⊥] Academy of Sciences of the Czech Republic.

10.1021/np0200360 CCC: $22.00
Published on Web 06/29/2002

Table 1. ^{1}H NMR Spectral Data [δ_H (J, Hz)] for Bufenolides **1**−**5**

position	1	2	3	4	5
1α	1.51 (2H)	1.51 (2H)	1.58	1.59	2.04
1β			1.38	1.37	1.47
2α	1.53 (2H)	1.54 (2H)	1.23	1.21	2.32
2β			1.85	1.85	2.21
3α	4.13 brt (2.3)	4.13 brt (2.3)	5.24 brs	5.23 s	
4α	1.88 td (13.8,2.8)	1.89	1.89	1.87	2.60
4β	1.34	1.35	1.48	1.46	2.04
5β	1.77	1.76	1.70	1.70	1.85
6α	1.21	1.21	1.53	1.52	1.31
6β	1.84	1.86	1.67	1.67	1.85
7α	0.95 qd (12.8,3.7)	0.93 qd (13.3,3.7)	0.93 qd (13.3,4.1)	0.91 qd (13.3,4.1)	1.00 qd (12.8,3.2)
7β	1.47	1.47	1.47	1.47	1.60
8β	1.99 td (11.9,3.7)	2.05 td (11.9,3.7)	2.00 td (11.9,3.7)	2.04	2.06
9α	1.57	1.57 td (11.5,3.7)	1.53	1.54	1.73 td (11.5,3.2)
11α	1.50	1.49	1.51	1.50	1.60
11β	1.30	1.32	1.30	1.33	1.42
12α	1.32	1.34	1.30	1.33	1.38
12β	1.60	1.70 dd (9.6,2.8)	1.60	1.69	1.67
15α	3.56 s	3.45 s	3.57 s	3.45 s	3.59 s
16α	2.27 (2H)	2.08	2.29 (2H)	2.10	2.30 (2H)
16β		1.89		1.89	
17α	1.46	1.77	1.45	1.77	1.50
18-Me	1.02 s	1.18 s	1.03 s	1.19 s	1.05 s
19-Me	0.99 s	1.00 s	1.00 s	1.02 s	1.05 s
21	5.30 s	5.40 s	5.30 s	5.40 s	5.31 d (0.9)
22	7.93 d (10.5)	7.69 brd (7.3)	7.93 dd (10.5,0.9)	7.69 brd (7.3)	7.93 dd (10.5,0.9)
23	6.03 d (10.5)	6.06 d (10.1)	6.04 d (10.1)	6.06 d (10.1)	6.05 d (10.1)
1′			8.07 s	8.07 s	

Table 2. ^{13}C NMR Spectral Data (125 MHz δ_C) for Bufenolides **1**−**5**

position	1	2	3	4	5
1	29.49	29.52	30.15	30.18	36.48
2	27.88	27.88	25.56	25.59	37.06
3	66.73	66.76	70.39	70.40	212.19
4	33.27	33.29	30.39	30.40	42.02
5	35.91	35.93	36.66	36.68	43.46
6	25.75	25.79	25.04	25.06	25.76
7	20.75	20.79	20.63	20.69	20.72
8	33.21	33.07	33.22	33.09	33.13
9	39.22	39.31	39.41	39.51	40.27
10	35.50	35.52	35.25	35.27	35.27
11	20.64	20.60	20.63	20.60	20.38
12	39.73	40.01	39.65	39.95	39.49
13	44.08	44.90	44.05	44.89	44.01
14	75.25	75.12	75.12	74.99	74.86
15	59.87	59.95	59.89	59.95	59.87
16	28.62	27.96	28.61	27.97	28.58
17	51.77	50.18	51.77	50.22	51.72
18	16.25	15.93	16.24	15.91	16.22
19	23.74	23.75	23.97	23.69	22.55
20	56.52	55.50	56.49	55.48	56.41
21	84.59	82.94	84.59	82.94	84.56
22	147.90	149.45	147.84	149.32	147.72
23	121.43	122.21	121.49	122.27	121.56
24	159.85	159.94	159.83	159.90	159.78
1′			160.69	160.69	

evidence that pyrone epoxide **1** possessed 24 carbon signals including five quaternary carbons, nine methines, eight methylenes, and two methyls. The ^{1}H NMR spectrum was similar to that of resibufogenin (**6**) and showed H-22 and H-23 signals (δ_H 7.93 and 6.03) characteristic of the bufadienolide 2-pyrone ring and the H-15 signal from the 14,15-epoxy group. However, the H-21 signal was absent and a new singlet signal was observed at δ_H 5.30, again indicating change in the 2-pyrone ring. Extensive change was also observed in the ^{13}C NMR spectrum. The C-20 and C-21 signals, which are usually observed at δ_C 120 and 150, respectively, had disappeared and new signals were observed at δ_C 56.52 and 84.59. These data suggested a 20,21-epoxy derivative of resibufogenin (**6**), and this was also

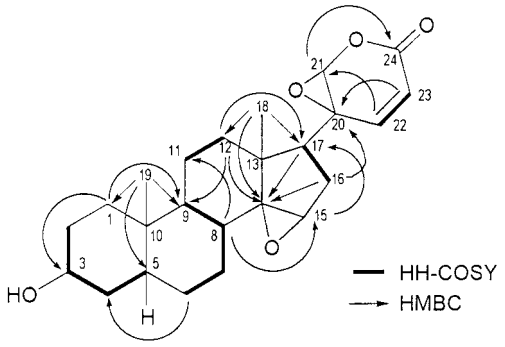


Figure 1. Selected 2D NMR correlations for bufenolide **1**.

supported by the molecular formula, which was increased by one oxygen atom. Analysis of the 2D NMR spectra was performed to determine the structure. The HH-COSY spectrum revealed the connectivities of C-2 to C-5, C-6 to C-9, C-11 to C-12, and C-16 to C-17, as shown in Figure 1. In the HMBC spectrum (Figure 1), HMBC correlations of H-1 to C-3 and C-9, H-6 to C-4, H-8 to C-11 and C-15, H-12 to C-9, C-14, and C-17, H-15 to C-17, H-16 to C-14, and H-17 to C-14 were observed. 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 quaternary carbons C-10 (δ_C 35.50) and C-13 (δ_C 44.08), respectively. These results allowed identification of the steroid skeleton of bufenolide **1**. The partial structure at C-17 was assigned by 2D NMR correlations as shown in Figure 1. The double bond between C-22 and C-23 was revealed by a correlation in the HH-COSY spectrum. HMBC correlations of H-23 to the new carbon signal at δ_C 56.52 and of H-22 to the other new signal at δ_C 84.59 in the HMBC spectrum suggested assignments as C-20 and C-21, respectively. These assignments were supported by an HMBC correlation of H-21 to C-24. The connectivity between C-17 and C-20 was suggested by HMBC correlations of H-16 to C-20. Thus, the structure was assigned as a 20,21-epoxyresibufogenin[6,7] structure with the 2-pyrone stereochemistry unknown.

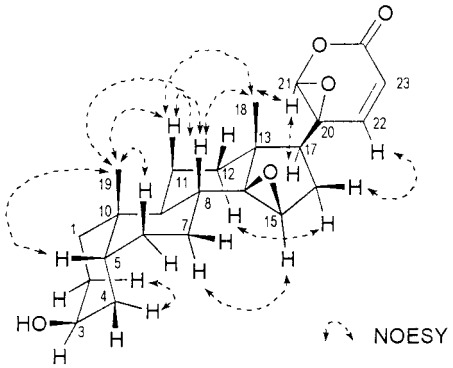


Figure 2. Selected NOESY correlations for bufenolide **1**.

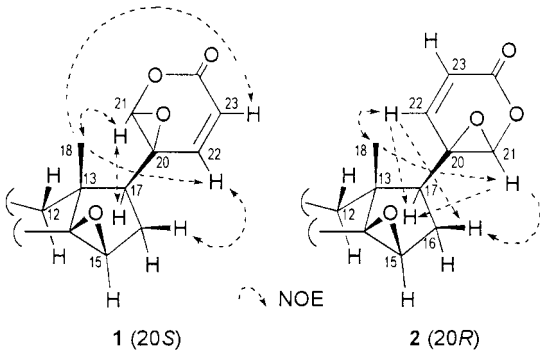


Figure 3. Difference NOEs of bufenolides **1** and **2**.

The relative stereochemistry of pyrone epoxide **1** was determined by the analysis of the phase-sensitive NOESY spectrum, which showed cross-peaks as shown in Figure 2. NOESY correlations of H-19/H-5, H-19/H-6β, H-19/H-8, and H-19/H-11β indicated that those protons were axial and β-oriented to the *cis*-ring junction at C-5 and C-10. A NOESY correlation of H-2/H-4α allowed assignment of the A-ring as in the chair conformation. Additional NOESY correlations of H-18/H-8 and H-18/H-11β as well as H-8/H-11β argued well for the stereochemistry of the B/C *trans* and C/D *cis* ring junctures and chair conformations for all six-membered rings. That was also supported by NOESY correlations of H-7α/H-15 and H-12α/H-16α. Thus, the relative stereostructure for the steroidal skeleton of bufenolide **1** was deduced as shown in Figure 2.

Pyrone epoxide **2** was obtained as colorless needles, and the UV and IR absorption (229 nm; 1744 cm^{-1}) again showed substantial change of the 2-pyrone ring. Analysis of NMR spectra including use of 2D NMR techniques led to the conclusion that compound **2** was also a 20,21-epoxyresibufogenin. However, bufenolides **1** and **2** had different R_f values on TLC (developed by 1:1 *n*-hexane−ethyl acetate) as well as opposite sign optical rotations ($[\alpha]^{18}_D$ +18° for **1** and $[\alpha]^{19}_D$ −17° for **2**). In addition, the ^{1}H NMR spectrum of epoxide **2** showed lower field shifts of H-17, H-18, and H-21 compared with those of epoxide **1** and a higher field shift of H-22 (Table 1). Furthermore, the H-22 signal was observed in broad doublet form for epoxide **2** (δ_H 7.69, brd, J = 7.3) and in doublet form for epoxide **1** (δ_H 7.93, d, J = 10.5). These data suggested that the stereochemistry of the epoxy group at C-20/C-21 differed.

Analysis of NOESY spectra was performed to assign the relative configuration of C-20 in the solution state. In the NOESY spectrum of epoxide **1**, correlations of H-21/H-17, H-21/H-18, and H-22/H-16β were observed as shown in Figure 2. On the other hand, the NOESY spectrum of **2** showed correlations of H-21/H-16β, H-21/H-17, H-22/H-17, and H-22/H-18. These NOESY correlations indicated that the relative configurations at C-20 were different and corresponded to the *S*-configuration for epoxide **1** and the *R*-configuration for epoxide **2**. We also examined the difference NOE spectra of epoxides **1** and **2** to confirm the configurations. Observed NOEs were as shown in Figure 3. In the difference NOE spectra of epoxide **1**, irradiation to H-18 provided new NOEs at H-22 and H-23 in addition to NOESY correlations as shown in Figure 2. This suggested that the relative configuration at C-20 was *S*. For epoxide **2**, new NOEs were observed at H-21 by irradiation to H-18 and at H-16β by irradiation to H-22, in addition to the NOESY correlations, and confirmed that C-20 corresponded to the *R*-configuration. Thus, the structures of epoxides **1** and **2** were elucidated to be 20*S*,21-epoxyresibufogenin and 20*R*,21-epoxyresibufogenin, respectively. The ^{1}H and ^{13}C NMR data have been summarized in Tables 1 and 2, respectively. As noted above, the isolation of a 20,21-epoxyresibufogenin had been reported and the overall structure had been determined.[8] However, only one 20,21-epoxyresibufogenin had been isolated, and the stereochemistry at C-20 had not been determined. The present study has led to isolation of two natural isomers (at C-20) of 20,21-epoxyresibufogenin and the first complete structural determinations.

Bufenolides **3** and **4** (colorless needles) corresponded to the molecular formula $C_{25}H_{32}O_6$ by HRFABMS [*m*/*z* 429.2302 $(M + H)^+$, Δ +2.5 mmu for **3** and *m*/*z* 429.2204 $(M + H)^+$, Δ −7.3 mmu for **4**]. The UV and IR absorption spectra showed considerable modifications of the 2-pyrone ring (232 nm; 1742 cm^{-1} for **3** and 229 nm; 1743 cm^{-1} for **4**). That was supported by the ^{1}H NMR spectra of epoxides **3** and **4**, which showed disappearance of H-21 signals, new singlet signals at δ_H 5.30 and 5.40, and spectral patterns similar to those of epoxides **1** and **2**, respectively (Table 1). Furthermore, the ^{1}H NMR spectra of epoxides **3** and **4** showed new singlet signals at δ_H 8.07, which were due to a formyl group, and low-field shifts of the H-3 signals for both, suggesting they were 3-formyl derivatives of epoxides **1** and **2**, respectively. The formyl signals correlated to C-3 in the HMBC spectra of epoxides **3** and **4**. Hence the structures were assigned as isomeric 3-*O*-formyl-20,21-epoxyresibufogenins.

The relative configurations at C-20 were expected to be *S* for **3** and *R* for **4** by the ^{1}H NMR spectral pattern and their optical rotations ($[\alpha]^{19}_D$ +17.2° for **3** and $[\alpha]^{19}_D$ −11.5° for **4**). Difference NOE spectral techniques confirmed that assumption. In the difference NOE spectra of **3**, irradiation of H-18 provided NOEs at H-21, H-22, and H-23, and NOEs at H-17 and H-18 were observed by irradiation to H-21. Also, irradiation to H-22 provided NOEs at H-16 and H-18, suggesting the relative configuration at C-20 of epoxide **3** was *S*. Conversely, epoxide **4** showed an NOE at H-21 by irradiation to H-18, NOEs at H-16β and H-17 by irradiation to H-21, and an NOE at H-18 by irradiation to H-22, indicating an *R*-configuration at C-20. That allowed the structures of epoxides **3** and **4** to be assigned 3-*O*-formyl-20*S*,21-epoxyresibufogenin and 3-*O*-formyl-20*R*,21-epoxyresibufogenin, respectively. The ^{1}H and ^{13}C NMR data are shown in Tables 1 and 2.

New bufenolide **5** (colorless needles) was determined to correspond to molecular formula $C_{24}H_{30}O_5$ by HRFABMS [*m*/*z* 399.2096 $(M + H)^+$, Δ −7.6 mmu]. The UV and IR absorptions (231 nm; 1742 cm^{-1}) suggested a 20,21-epoxybufenolide system, and this was supported by the ^{1}H NMR spectrum, which showed an absence of an H-21 signal and a new signal at δ_H 5.31. In addition, the ^{1}H NMR

Table 3. Cancer Cell Line Evaluation of Compounds **1**−**6**

compd no.	IC_{50} (μg/mL) KB[a]	MH-60[b]
1	10.88	1.82
2	8.09	1.80
3	>25	>25
4	>25	>25
5	18.51	8.54
6	1.34	10.48

[a] Human oral epidemoid carcinoma cells. [b] Murine leukemia cells.

spectrum was similar to that of epoxide **1**, but with no H-3 signal. Analogous differences were also observed in the ^{13}C NMR spectrum: the disappearance of the hydroxymethine carbon signal at C-3 and the appearance of a new quaternary carbon signal at δ_C 212.2. These observations indicated that epoxide **5** was the 3-oxo derivative of epoxide **1**, and this was supported by two fewer hydrogen atoms in the molecular formula. HMBC correlations of H-2 and H-4 to the quaternary carbon signal at δ_C 212.2 provided the assignment 3-oxo-20,21-epoxyresibufogenin (**5**). The relative configuration at C-20 was expected to be *S* by the ^1H NMR spectral pattern and was confirmed by difference NOE spectra, which showed NOEs at H-21 and H-22 by irradiation to H-18, an NOE at H-17 by irradiation to H-21, and an NOE at H-18 by irradiation to H-22. Therefore, the structure of epoxide **5** was determined to be 3-oxo-20*S*,21-epoxyresibufogenin. The interpreted ^1H and ^{13}C NMR data are displayed in Tables 1 and 2.

The structures of bufenolides **1**−**5** were further substantiated by semisynthetic procedures. Epoxidation of steroidal olefins is usually achieved by using a peracid such as *m*-chloroperbenzoic acid. Because of its pseudoaromaticity, this reagent does not attack the 2-pyrone ring, and instead we utilized the Kimura[9] epoxidation with hydrogen peroxide-tris(acetylacetonato) iron(III). Epoxidation of resibufogenin (**6**) in acetonitrile at 0 °C for 1.5 h afforded a pair of 20,21-epoxy derivatives corresponding to bufadienolides **1** and **2**. The epoxides were separated by SiO_2 column chromatography and were found to be identical with bufenolides **1** and **2** isolated from Ch'an Su.

Syntheses of **3**, **4**, and **5** were completed by formylation or oxidation of the appropriate 20,21-epoxyresibufogenin. Formates **3** and **4** were prepared by treatment of epoxides **1** and **2**, respectively, with formic acid in pyridine. Oxidation of epoxide **1** with chromium(VI) oxide in acetic acid provided a good yield of a 3-ketone which was identical with epoxide **5**.

The cancer cell growth inhibition effects of epoxides **1**−**6** against human oral epidermoid carcinoma KB cells and murine leukemia MH-60 cells are shown in Table 3. Bufadienolide **6** showed more activity against KB cells than against the MH-60 cells. On the other hand, 20,21-epoxybufenolides **1**, **2**, and **5** were more inhibitory of MH-60 cells than KB cells. Formates **3** and **4** were inactive against both cancer lines. The result was quite useful for structure−activity relationship (SAR) purposes and shows that 20,21-epoxybufenolides may generally exhibit less and/or different activity than the parent bufadienolide system. Other types of biological activities will need to be assessed for such new 20,21-epoxybufenolides.

Experimental Section

General Experimental Methods. Thin-plate Ch'an Su was obtained in a Hong Kong folk-medicinal market.[2a] Other materials, reagents, instruments, and experimental procedures have been summarized in the preceding contribution.[2a]

Extraction and Isolation. Thin-plate Ch'an Su (1.0 kg) was ground into a rough powder and extracted (3×) with CH_2Cl_2 for a week at room temperature. The extract was concentrated under reduced pressure, and the residue (250 g) was subjected to column chromatography on silica gel, eluting with a *n*-hexane−acetone gradient (increasing acetone 0−100%). Five fractions (F1−F5) were obtained, and fraction 2 (expected to be less polar bufadienolides from TLC developed by 4:3:3 *n*-hexane−CH_2Cl_2−acetone) was subjected to column chromatography on silica gel, again eluting with the *n*-hexane−acetone system by increasing acetone 0−100%. Another five fractions (F6−F10) were selected, and a part of fraction 8 (8.9 g) was rechromatographed on silica gel eluting with a *n*-hexane−ethyl acetate gradient by increasing ethyl acetate 10−100% to provide five fractions (F11−F15). Fractions 12, 13, and 14 were rechromatographed on silica gel and Sephadex LH-20 repeatedly, the TLC color reactions being noted. Fractions 12, 13, and 14 gave 20*S*,21-epoxyresibufogenin (**1**, 444.2 mg), 20*R*,21-epoxyresibufogenin (**2**, 310.9 mg), and resibufogenin (**6**), respectively. Fraction 7 was submitted to column chromatography on silica gel eluting with the *n*-hexane−ethyl acetate gradient system (increasing ethyl acetate 0−100%) to provide six fractions (F16−F21). Fractions 17 and 19 were rechromatographed on silica gel and Sephadex LH-20 repeatedly with TLC color reaction surveillance. As a result, 3-*O*-formyl-20*S*,21-epoxyresibufogenin (**3**, 21.9 mg) and 3-*O*-formyl-20*R*,21-epoxyresibufogenin (**4**, 8.2 mg) were obtained from fraction 17, and fraction 19 gave 3-oxo-20*S*,21-epoxyresibufogenin (**5**, 47.8 mg).

20*S*,21-Epoxyresibufogenin (1): colorless plates; mp 184−186 °C; $[\alpha]^{18}_D$ +18.2° (*c* 0.1, $CHCl_3$); UV (CH_3OH) λ_{max} (log ϵ) 233 nm (3.5); IR (KBr) ν_{max} 3465, 3070, 2937, 2877, 1744, 1625, 1533, 1123, 1089, 982, 886, 785 cm^{-1}; FABMS *m*/*z* 401 $[M + H]^+$; HRFABMS *m*/*z* 401.2252 [calcd for $C_{24}H_{23}O_5$ $(M + H)^+$, 401.2328]; ^1H and ^{13}C NMR data are shown in Tables 1 and 2, respectively.

20*R*,21-Epoxyresibufogenin (2): colorless needles; mp 90−94 °C; $[\alpha]^{19}_D$ −17.0° (*c* 0.1, $CHCl_3$); UV (CH_3OH) λ_{max} (log ϵ) 229 nm (3.5); IR (KBr) ν_{max} 3470, 3070, 2938, 2877, 1744, 1632, 1534, 1124, 1093, 980, 875, 782 cm^{-1}; FABMS *m*/*z* 401 $[M + H]^+$; HRFABMS *m*/*z* 401.2271 [calcd for $C_{24}H_{33}O_5$ $(M + H)^+$, 401.2328]; Tables 1 and 2 for ^1H and ^{13}C NMR data.

20*S*,21-Epoxyresibufogenin 3-formate (3): colorless needles; mp 180−182 °C; $[\alpha]^{19}_D$ +17.2° (*c* 0.1, $CHCl_3$); UV (CH_3CN) λ_{max} (log ϵ) 232 nm (3.8); IR (KBr) ν_{max} 3031, 2939, 2880, 1742, 1540, 1448, 1374, 1149, 1120, 1092, 984, 868, 839 cm^{-1}; FABMS *m*/*z* 429 $[M + H]^+$; HRFABMS *m*/*z* 429.2302 [calcd for $C_{25}H_{33}O_6$ $(M + H)^+$, 429.2277]; ^1H and ^{13}C NMR data (Tables 1 and 2).

20*R*,21-Epoxyresibufogenin 3-formate (4): colorless needles; mp 147−150 °C; $[\alpha]^{19}_D$ −11.5° (*c* 0.1, $CHCl_3$); UV (CH_3CN) λ_{max} (log ϵ) 229 nm (3.7); IR (KBr) ν_{max} 3648, 2940, 2364, 1743, 1716, 1541, 1507, 1457, 1193, 1152, 1092, 983, 882 cm^{-1}; FABMS *m*/*z* 429 $[M + H]^+$; HRFABMS *m*/*z* 429.2204 [calcd for $C_{25}H_{33}O_6$ $(M + H)^+$, 429.2277]; ^1H and ^{13}C NMR data appear in Tables 1 and 2.

3-Oxo-20*S*,21-epoxyresibufogenin (5): colorless needles; mp 180−182 °C; $[\alpha]^{20}_D$ +30.8° (*c* 0.1, $CHCl_3$); UV (CH_3OH) λ_{max} (log ϵ) 231 nm (3.6); IR (KBr) ν_{max} 3778, 2936, 2867, 1742, 1708, 1654, 1637, 1450, 1223, 1122, 1088, 983, 866, 827 cm^{-1}; FABMS *m*/*z* 399 $[M + H]^+$; HRFABMS *m*/*z* 399.2096 [calcd for $C_{24}H_{31}O_5$ $(M + H)^+$, 399.2172]; ^1H and ^{13}C NMR data (Tables 1 and 2).

Syntheses of 20*S*,21-Epoxyresibufogenin and 20*R*,21-Epoxyresibufogenin. Resibufogenin (**6**, 0.31 g) and 2.5 g of tris(acetylacetonato)iron(III) were dissolved in acetonitrile (250 mL), and 30% aqueous hydrogen peroxide was slowly added (18.0 mL dropwise) at 0 °C for 1.5 h. After filtration of the mixture, the filtrate was poured into ice−water and the aqueous phase was extracted with CH_2Cl_2. The organic extract was washed with dilute aqueous sodium thiosulfate, aqueous sodium hydrogen carbonate, and water (100 mL each), dried (magnesium sulfate), and concentrated under reduced pressure. The residue (0.28 g) was separated by column chromatography on silica gel, eluting with the *n*-hexane−ethyl

acetate gradient system (increasing ethyl acetate from 10 to 60%) to afford colorless needles (**1**, 80 mg) first, followed by epoxide **2** as colorless needles (43 mg). Their spectral data corresponded with those of natural epoxides **1** and **2**, respectively.

Syntheses of 3-*O*-Formyl-20*S*,21-epoxyresibufogenin (3) and 3-*O*-Formyl-20*R*,21-epoxyresibufogenin (4). A mixture of 20*S*,21-epoxyresibufogenin (0.10 g) and formic acid (2.2 mL) in pyridine (2.0 mL) was stirred for 22 h at room temperature. After extraction with CH_2Cl_2 and removal of the solvent, the residue (90 mg) was recrystallized from acetone to give colorless needles (**3**, 75 mg), with spectral data identical to those of natural product **3**. By the same method, starting with 20*R*,21-epoxyresibufogenin, 3β-formate **4** (colorless needles, 70 mg) was synthesized and its spectral data corresponded to those of natural formate **4**.

Synthesis of 3-Oxo-20*S*,21-epoxyresibufogenin. To a solution of 20*S*,21-epoxyresibufogenin (0.10 mg) in acetic acid (1.5 mL) was added a solution of chromium(VI) oxide (55 mg) in acetic acid (1.0 mL) with stirring. The oxidation was conducted at room temperature for 5 h. After decomposition of the excess reagent with methanol (0.25 mL, with stirring), the mixture was poured into ice−water and concentrated to dryness. Recrystallization of the crude product from methanol gave colorless needles (**5**, 70 mg) identical (spectral data) to the natural product (**5**).

Biological Activities. Human oral epidemoid carcinoma KB cells were maintained in culture flasks in MEM (modified Eagle medium) supplemented with 10% FBS (fetal bovine serum); murine leukemia cells were maintained in RPMI 1640 medium supplemented with kanamaycin sulfate (100 μg/mL). For the in vitro drug treatment experiments, tumor cells (2×10^4 cells for KB cells and 5×10^3 cells for MH-60 cells) were seeded in 0.2 mL of culture medium/well in 96-well plates (Corning Glass Works). The cells were treated in triplicate with graded concentrations of 5 μL test samples and were then incubated in a 5% carbon dioxide atmosphere at 37 °C for 72 h. The 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay was used to measure the cytotoxic effect. The activity was shown as the IC_{50} value, which was the concentration (μg/mL) of test compound to give 50% inhibition of cancer cell line growth.

Acknowledgment. The authors thank H. Yokoyama of Kanagawa University for the measurements of NMR spectra; the Outstanding Investigator Award (to G.R.P.) CA-4434401-2A1 and 1RO1-CA-90441-01 from the Division of Cancer Treatment and Diagnosis, NCI, DHHS; and F. Hogan for other very helpful assistance.

References and Notes

(1) Iizuka, M.; Warashina, T.; Noro, T. *Chem. Pharm. Bull.* **2001**, *49*, 282−286.
(2) (a) Nogawa, T.; Kamano, Y.; Yamashita, A.; Pettit, G. R. *J. Nat. Prod.* **2001**, *64*, 1148−1152. (b) Matsukawa, M.; Akizawa, T.; Ohigashi, M.; Morris, J. F.; Butler, V. P., Jr.; Yoshioka, M. *Chem. Pharm. Bull.* **1997**, *45*, 249−254. (c) Rossi, M. H.; Blumentahl, E. E. A.; Jared, C. *An. Assoc. Bras. Quim.* **1997**, *46*, 21−26. (d) Matsukawa, M.; Akizawa, T.; Morris, J. F.; Butler, V. P., Jr.; Yoshioka, M. *Chem. Pharm. Bull.* **1996**, *44*, 255−257.
(3) (a) Pettit, G. R.; Green, B.; Dunn, G. L. *J. Org. Chem.* **1970**, *35*, 1367−1376. (b) Pettit, G. R.; Knight, J. C.; Herald, C. L. *J. Org. Chem.* **1970**, *35*, 1393−1398. (c) Pettit, G. R.; Fessler, D. C.; Paull, K. D.; Hofer, P.; Knight, J. C. *J. Org. Chem.* **1970**, *35*, 1398−1404. (d) Kamano, Y.; Pettit, G. R.; Inoue, M. *J. Org. Chem.* **1974**, *39*, 3007−3010. (e) Kamano, Y.; Pettit, G. R.; Tozawa, M.; Komeichi, Y.; Inoue, M. *J. Org. Chem.* **1975**, *40*, 2136−2138.
(4) (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) Kawahara, K.; Mikage, M. *Yakugaku Zasshi* **2000**, *120*, 1217−1219. (c) Kamano, Y. *Kagaku no Ryouiki* **1970**, *24*, 339 and 421.
(5) Kamano, Y.; Nogawa, T.; Kotake, A.; Tozawa, M.; Pettit, G. R. *J. Liq. Chrom., Relat. Technol.* **1999**, *22*, 2455−2465.
(6) Kamano, Y.; Pettit, G. R.; Inoue, M.; Tozawa, M.; Smith, C. R.; Weisleder, D. *J. Chem. Soc., Perkin Trans. 1* **1988**, 2037−2041.
(7) Komatsu, M.; Kamano, Y.; Suzuki, M. *Jpn. Analyst* **1965**, *14*, 1049−1054.
(8) Kaneda, N.; Kuraishi, T.; Yamasaki, K. *Chem. Pharm. Bull.* **1981**, *29*, 257−259.
(9) (a) Tohma, M.; Tomita, T.; Kimura, M. *Tetrahedron Lett.* **1973**, *44*, 4362. (b) Yamamoto, M.; Kimura, M. *J. Chem. Soc., Chem. Commun.* **1977**, 948−949.

NP0200360