RESEARCH ARTICLE

Bufadienolides with cytotoxic activity from the eggs of *Bufo bufo gargarizans*

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ABSTRACT

Seven bufadienolides (1–7) were isolated from the eggs of toad *Bufo bufo gargarizans*. Their chemical structures were identified on the basis of various spectroscopic methods, including NMR and HRESIMS. These compounds were tested for their cytotoxic effects against human lung cancer cell line A549, human liver cancer cell line BEL-7402 and human gastric cancer cell line BGC-823. All of them exhibited potent cytotoxic activity.

KEYWORDS *Bufo bufo gargarizans*, eggs, bufadienolide, cytotoxic effects

INTRODUCTION

Bufadienolide is a kind of compound with steroidal skeleton and possesses a characteristic α-pyrone ring at its C-17β position, which is a class of interesting natural products with various pharmacological activities[1]. So far, this kind of compounds have been found and obtained by the method of isolation from many plant and animal families, biotransformation and synthesis[2,3]. According to modern pharmacological research on bufadienolides, it has been revealed that they possess cardiac activity, anti-cancer activity, local anaesthesia effect, analgesic effect, anti-inflammatory effect and so on[4,5].

Bufo bufo gargarizans is a species of genus *Bufo* belonging to family Bufonidae and widely distributed in China, Russian and North Korea[6]. It has reported that the toad is a gold mine on account of bufadienolides being widely distributed all over its body, such as toad venom, toad skin, and toad bile[7], which play an important role in therapies of many diseases in China. Taking Chinese traditional medicine, toad venom for example, toad venom is also called *Chan'su*, which contains various and numerous bufadienolides, and it has recorded that it has potent anti-tumor, local anaesthesia, analgesic effects.

The eggs of *Bufo bufo gargarizans* are the eggs of female *Bufo bufo gargarizans*'s ovaries[8]. Previous researches discovered that the eggs of toad possess abundant and specific bufadienolides[1]. Therefore, our study focused on the chemical constituent research of 95% ethanol extract fraction from the eggs of toad *Bufo bufo gargarizans*. Seven bufadienolides (**1–7**) were isolated and identified from the eggs of toad *Bufo bufo gargarizans* (Fig. 1), including cinobufagin (**1**)[9], bufalin (**2**)[10], bufotalinin (**3**)[11], bufotalin (**4**)[11], marinobufagenin (**5**)[12], hellebrigenol (**6**)[13], arenobufagin (**7**)[14]. Since bufadienolides has become a promising anticancer prototypes[15], we further tested their cytotoxic effects against human lung cancer cell line A549, human liver cancer cell line BEL-7402, and human gastric cancer cell line BGC-823. BGC-823 cancer cell line was performed on bufadienolides for the first time.

MATERIALS AND METHODS

General experimental procedures

HRESIMS spectra were measured on an Agilent 6210 HPLC/MS TOF mass spectrometer (Agilent Technologies, Santa Clara, CA, USA). UV spectra were recorded on a JASCO V-550 UV/VIS spectrometer (JASCO Corporation, Tokyo, Japan). IR spectra were carried out on a JASCO FT-IR-4600 infrared spectrometer (JASCO Corporation, Tokyo, Japan). All the NMR spectra were acquired on a Bruker AV-300 spectrometer (Bruker Instrument, Inc., Zurich, Switzerland). HPLC was performed on an Agilent 1200 HPLC system equipped with a diode

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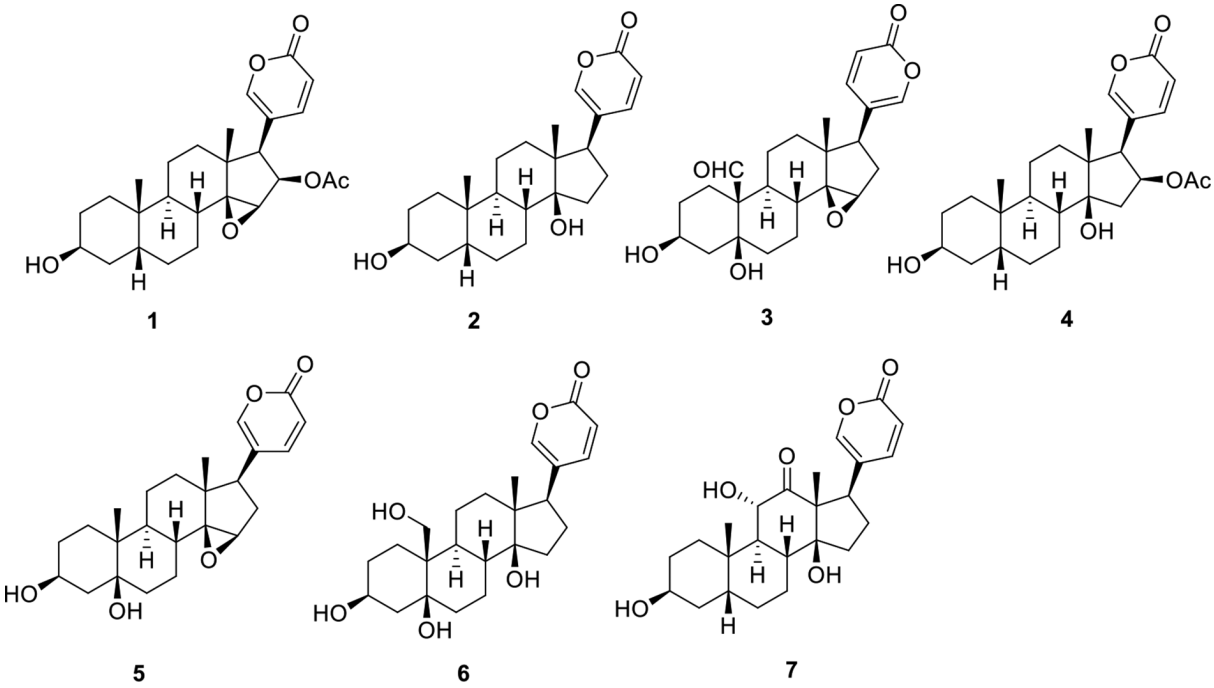

Fig. 1 Chemical structures of compounds (1–7).

array detector, using a biphenyl column (Kinetex Biphenyl, 5 μm, 4.6 × 250 mm, Phenomenex, USA) for analysis. Pre-HPLC was conducted on a Wufeng L-100 HPLC system (Wufeng Corporation, Shanghai, China), using a biphenyl preparative column (Kinetex Biphenyl, 10 μm, 10 × 250 mm, Phenomenex, USA) for preparative purification. Open column chromatography (CC) was operated on reverse-phase C18 silica gel (YMC Corporation, Japan) and silica gel (200–300 mesh, Haiyang Chemical Group Corporation, Qingdao, China).

Materials

The eggs of *Bufo bufo gargarizans* Cantor were obtained from Dongtai Toad Breeding farm in Jiangsu province of China between March and May 2015, and authenticated by Professor Guang-Xiong Zhou (Jinan University). A specimen (No. 201503031) was deposited in the Institute of Traditional Chinese Medicine and Natural Products, College of Pharmacy, Jinan University, China.

Human cell lines A549, BEL-7402 and BG-823 were obtained from the American Type Culture Collection (ATCC) and were maintained in RMPI 1640 Medium containing 10% fetal bovine serum (NBS), 100 IU/ml penicillin and 100 mg/ml streptomycin in humidified 5% CO_2 atmosphere at 37°C as previously described.

Isolation of bufadienolides

The dried and powdered eggs of *Bufo bufo gargarizans* (25 kg) were extracted by 95% EtOH under ultrasonic condition (40 min, 40°C) three times. After removal of the solvent extract under reduced pressure, the crude extract (2.5 kg) was suspended in 20% EtOH (20 L) and then partitioned by hexamethylene (3 × 20 L) and EtOAc (3 × 20 L). The concentrated ethyl acetate layer (253 g) was mainly subjected to silica gel (200–300 mesh), eluted with dichloromethane-methanol (100:1, 50:1, 20:1 and 10:1) to give 10 fractions (Fr.1-16). Fr.3 (1.6 g), Fr.6 (2.6 g), Fr.7 (2.3 g) was further separated by reverse-phase C18 silica using methanol–water gradients and Pre-HPLC. Compounds **1** (25.1 mg) and **2** (15.9 mg) were obtained from Fr.3. Compounds **3** (9.9 mg) and **4**(10.9 mg) were obtained from Fr.6. Compounds **5** (1.2 mg), **6** (12.9 mg) and **7** (11.6 mg) were obtained from Fr.7.

Cytotoxic assay

Cell viability assay was completed according to the protocol of Cell Titer-Glo Luminescent kit (Promega). In brief, 5000 cells were seeded on 96-well plates in culture medium, 100 μl per well for 24 h. Prepare control wells containing medium without cells to obtain a value for background luminescence. The test compounds (**1–7**) were added to experimental wells, and incubated for additional 48 h. Add a volume of Cell Titer-Glo reagent equal to the volume of cell culture medium present in each well (e.g., add 100 μl of reagent to 100 μl of medium containing cells for a 96-well plate), Mix contents for 2 min on an orbital shaker to induce cell lysis. Allow the plate to incubate at room temperature for 10 min to stabilize the luminescent signal. Luminescence signal was recorded by BIOTEK FLX800. Data were analyzed by Graph pad prism 5 and expressed in mean ± SD.

RESULTS AND DISCUSSION

Compounds **1–7** were obtained by isolation and identification of extract from the eggs of *Bufo bufo gargarizans*, and they had potential cytotoxic activity.

Spectradata of 1–7

Compound **1** was obtained as white powder. UV (MeOH) λ_{max} nm (log ε): 204 (3.94), 298 (3.71). IR (KBr) ν_{max}: 3432, 2936, 2847, 1714, 1535, 1034 cm^{-1}. HRESIMS *m/z:* 401.2320 (calculated for $C_{24}H_{34}O_4$, 401.2320 $[M+H]^+$). ^1H NMR (CD_3OD, 300 MHz): δ_H 0.71 (3H, s, H-18), 0.96 (3H, s, H-19), 4.06(1H, br, s, H-3), 6.28 (1H, d, J = 9.7 Hz, H-23), 7.43(1H, d, J = 2.6 Hz, H-21), 8.00 (1H, dd, J = 9.7, 2.6 Hz, H-22), ^{13}C NMR(CD_3OD, 75 MHz) data, see Table 1.

Compound **2** was also obtained as white powder. UV (MeOH) λ_{max} nm (log ε): 203 (log ε 2.74), 294 (log ε 2.53). IR (KBr) ν_{max}: 3494, 1724, 1634, 1536, 1451, 1378, 1244, 1123, 1036, 982, 954, 907, 882, 837, 790 cm^{-1}. HRESIMS *m/z:* 443.2432 (calculated for $C_{26}H_{34}O_6$, 443.2428 $[M+H]^+$). ^1H NMR (CD_3OD, 300 MHz): δ_H 0.82 (3H, s, H-18), 1.00 (3H, s, H-19), 4.05(1H, br, s, H-3), 6.25 (1H, d, J = 9.8 Hz, H-23), 7.37(1H, d, J = 2.5 Hz, H-21), 8.03 (1H, dd, J = 9.8, 2.6 Hz, H-22), ^{13}C NMR (CD_3OD, 75 MHz) data, see Table 1.

Compound **3** was obtained as amorphous powder. UV (MeOH) λ_{max} nm (log ε): 203 (3.73), 296 (3.61). IR (KBr) ν_{max}: 3419, 2940, 1708, 1129 cm^{-1}. HRESIMS *m/z:* 415.2116 (calculated for $C_{24}H_{30}O_6$, 415.2115 $[M+H]^+$). ^1H NMR (CD_3OD, 300 MHz): δ_H 0.61 (3H, s, H-18), 3.99(1H, br, s, H-3), 6.24 (1H, d, J = 9.8 Hz, H-23), 7.50(1H, d, J = 2.5Hz, H-21), 7.72 (1H, dd, J = 9.8, 2.6 Hz, H-22), ^{13}C NMR (CD_3OD, 75 MHz) (Table 1).

Compound **4** was obtained as colorless needle-like crystal. UV (MeOH) λ_{max} nm (log ε): 204 (3.97), 296 (3.77). IR (KBr) ν_{max}: 3414, 2918, 1721, 1540, 1254, 1038 cm^{-1}. HRESIMS *m/z*: 445.2586 (calculated for $C_{26}H_{34}O_6$, 445.2586 $[M+H]^+$). ^1H NMR (CD_3OD, 300 MHz): δ_H 0.78 (3H, s, H-18), 0.96 (3H, s, H-19), 4.06 (1H, br, s, H-3), 6.21 (1H, d, J = 9.7 Hz, H-23), 7.44

Table 1 ^{13}C NMR spectral data of Compounds 1–7 (δ in ppm, measured in CD_3OD, 75 MHz).

No.	1	2	3	4	5	6	7
1	29.9	30.7	17.0	30.8	24.9	20.1	33.0
2	27.9	28.5	26.8	28.5	28.0	28.2	29.3
3	67.7	67.6	65.6	67.7	68.1	68.8	67.7
4	33.2	34.5	37.4	34.1	37.0	38.0	33.2
5	36.5	36.5	73.9	36.6	74.8	78.8	39.2
6	28.6	26.9	35.3	27.8	34.7	36.8	27.8
7	22.6	21.6	22.8	22.3	23.4	25.0	22.8
8	43.0	34.0	41.3	43.0	32.7	41.8	40.8
9	36.9	40.7	33.3	37.4	42.8	40.1	39.2
10	36.5	37.3	54.6	36.4	41.0	43.6	38.2
11	22.6	22.1	21.8	22.3	21.6	23.0	75.0
12	42.0	40.2	39.4	41.1	39.5	42.1	215.0
13	49.6	46.3	44.4	50.8	45.2	49.8	63.8
14	86.2	73.5	73.9	85.1	74.8	86.1	86.2
15	34.2	60.8	59.4	41.3	60.0	32.8	27.8
16	27.2	76.6	31.5	75.7	32.4	29.7	34.6
17	52.3	51.4	45.9	58.2	47.7	52.0	42.0
18	17.3	17.6	17.6	17.1	16.9	17.2	18.0
19	24.3	24.2	209.0	20.9	16.9	65.9	24.0
20	125.1	118.5	121.9	119.4	122.3	124.9	123.2
21	150.4	153.5	150.6	152.1	150.0	150.5	151.6
22	149.4	150.8	147.4	152.8	147.1	149.3	149.1
23	115.4	114.1	114.2	113.1	115.4	115.5	115.9
24	164.8	163.9	161.1	164.4	162.2	164.8	164.4
$COCH_3$		20.4		24.3			
$COCH_3$		171.5		171.9			

Table 2 The IC_{50} values of compounds 1–7 against A549, BEL-7402 and BGC-823 cell lines.

Compound	IC_{50} (μM)		
	A549	BEL-7402	BG-823
1	0.004494 ± 0.003192	0.008863 ± 0.000956	0.013207 ± 0.001572
2	0.010467 ± 0.001121	0.016240 ± 0.001623	0.034210 ± 0.003960
3	0.594267 ± 0.014379	0.791900 ± 0.010583	1.105667 ± 0.015308
4	0.035383 ± 0.004875	0.059553 ± 0.003456	0.118933 ± 0.008162
5	0.632367 ± 0.058982	1.827667 ± 0.167375	2.700333 ± 0.027025
6	0.014227 ± 0.002628	0.023410 ± 0.005112	0.038673 ± 0.001850
7	0.006739 ± 0.001847	0.008743 ± 0.000525	0.011587 ± 0.000843

All data are presented as means ± SD of at three independent experiments.

(1H, d, J = 2.4Hz, H-21),8.26 (1H, dd, J = 9.7, 2.4 Hz, H-22), ^{13}C NMR(CD_3OD, 75 MHz) (Table 1).

Compound **5** was obtained as white powder. UV(MeOH) λ_{max} nm (log ε): 204 (3.97), 296 (3.77). IR (KBr) ν_{max}: 341.4, 2918, 1721, 1540, 1254, 1038 cm^{-1}. HRESIMS *m/z*: 401.2323 (calculated for $C_{24}H_{32}O_5$, 401.2320[M+H]$^+$). ^{1}H NMR (CD_3OD, 300 MHz): δ_H 0.77 (3H, s, H-18),0.97 (3H, s, H-19), 4.17(1H, br s, H-3), 6.24 (1H, d, J = 9.8 Hz,H-23),7.23(1H, d, J = 2.6 Hz, H-21), 7.77 (1H, dd, J = 9.8, 2.6 Hz, H-22), ^{13}C NMR (CD_3OD, 75 MHz) (Table 1).

Compound **6** was obtained as white powder. UV (MeOH) λ_{max} nm (log ε): 204 (3.99), 296 (3.69). IR(KBr) ν_{max}: 3412, 2938, 1710, 1632, 1538, 1451, 1138 cm^{-1}. HRESIMS *m/z*: 419.2434 (calculated for $C_{23}H_{32}O_6$, 419.2428[M+H] $^+$). ^{1}H NMR (CD_3OD, 300 MHz):δ_H 0.72 (3H, s, H-18), 4.12 (1H, br, s, H-3), 6.30 (1H, d, J = 9.7 Hz, H-23), 7.44 (1H, d, J = 2.5 Hz, H-21), 8.00 (1H, dd, J = 9.7, 2.5 Hz, H-22), ^{13}C NMR (CD_3OD, 75 MHz) (Table 1).

Compound **7** was obtained as colorless black crystal. UV (MeOH) λ_{max} nm (log ε): 204 (3.91), 298 (3.67). IR (KBr) ν_{max}: 3384, 2947, 1706, 1694, 1537 cm^{-1}. HRESIMS *m/z*: 417.2271 (calculated for $C_{23}H_{32}O_6$, 417.2271[M+H]$^+$). ^{1}H NMR (CD_3OD, 300 MHz): δ_H 0.91 (3H, s, H-18), 1.18 (3H, s, H-19), 4.37(1H, br, s, H-3), 6.31 (1H, d, J = 9.8 Hz, H-23), 7.52(1H, d, J = 2.6 Hz, H-21), 7.91 (1H, dd, J = 9.8, 2.6 Hz, H-22), ^{13}C NMR (CD_3OD, 75 MHz) (Table 1).

Cytotoxic activity

Bufadienolides have attracted much attention on account of their anticancer effects. To further investigate the antitumor activity, the cytotoxic effects of Compounds **1–7** against human lung cancer cell line A549, human liver cancer cell line BEL-7402 and human gastric cancer cell line BGC-823 by the Cell Titer-Glo luminescent cell viability assay method[16]. According to the results, all the compounds showed powerful cytotoxic activity against all cell lines especially cancer cells A549 (Table 2). Compounds **1** and **7**even exhibited lower IC_{50} values less than 0.01 μM.

CONCLUSION

To conclude, seven bufadienolides (**1–7**) were isolated and identified from the eggs of *Bufo bufo gargarizans* via various modern isolated approaches and identified methods, which exhibited a good result on the cytotoxic activity assay. Thus, our work provides a convinced proof for further research on antitumor products.

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