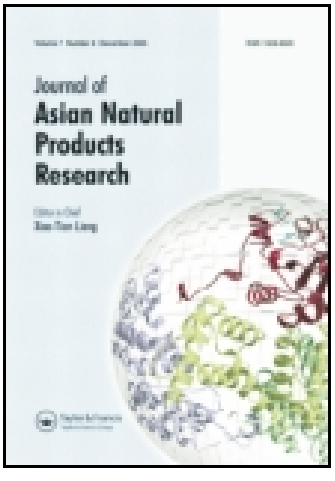

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Cytotoxic constituents from the skin of the toad *Bufo bufo gargarizans*

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To study the chemical composition of the skin of *Bufo bufo gargarizans*, various chromatographic methods were used in the isolation procedures and the structures of isolated compounds were determined based on NMR and MS analysis. As a result, two new compounds were isolated from its ethanolic extract and characterized as *N*-[2-hydroxy-2-(4-hydroxyphenyl)ethyl]-*N*-methylurea (**1**) and 19-oxocinobufotalin 3-adipoylarginine ester (**2**), together with 11 known compounds. Isolated bufadienolides showed significant inhibition effect against human hepatocellular carcinoma cell line SMMC-7721 *in vitro*.

Keywords: *Bufo bufo gargarizans*; toad skin; *N*-[2-hydroxy-2-(4-hydroxyphenyl)ethyl]-*N*-methylurea; 19-oxocinobufotalin 3-adipoylarginine ester; cytotoxicity

1. Introduction

Toads, particularly members of the genus *Bufo*, are identified as a convenient and useful source of granular gland secretions that commonly contain biogenic amines, bufodienolides, alkaloids, steroids, peptides, and proteins. Including genus *Bufo*, the different amphibian skin possessed a wide range of biologically active substances with a therapeutic potential. In the traditional Chinese medicine, amphibian skin had been used for the alleviation of human sufferings. Chan'Pi, the skin of a Chinese toad (*Bufo bufo gargarizans*), whose extract preparation known as 'Hua Chan Su' was used in the treatment of various diseases, including cancer, arrhythmia, and heart diseases [1]. Many of these effects are attributed to bufadienolides [2], one of the active compounds in this drug. Recently, new biological activities of bufadienolides, such as the effect of bufalin on inducing apoptosis in human leukemia U937 cells by modulating the mitogen-activated protein kinase pathways, the inhibitory effect of several bufadienolides on tumor cell growth, and the inhibitory effect on the IL-6 activities by 20*S*,21-epoxy resibufogenin, have been reported [3]. In order to find new and bioactive bufadienolides from Chan'Pi, we investigated the skin of *B. b. gargarizans*. In our previous studies, two new bufadienolides with cytotoxic activity were isolated [4]. We obtained another two new compounds from its ethanolic extract, named *N*-[2-hydroxy-2-(4-hydroxyphenyl)ethyl]-*N*-methylurea (**1**) and 19-oxocinobufotalin 3-adipoylarginine ester (**2**), together with 11 known compounds. Moreover, the isolated bufadienolides showed significant inhibition effect against human hepatocellular carcinoma cell line SMMC-7721 *in vitro.*

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2. Results and discussion

Compound **1** was isolated as a white powder. ESI-MS gave its quasi-molecular ion at *m/z* 211 $[M + H]^+$, corresponding to the molecular formula $C_{10}H_{14}N_2O_3$ in the HR-ESI-MS measurement. The ^1H NMR spectrum (DMSO-d_6, 400 MHz) of **1** showed the presence of *para*-substituted benzene ring signals at δ 7.13 (2H, d, $J = 8.4$ Hz) and 6.73 (2H, d, $J = 8.4$ Hz) as well as a signal at δ 2.70 (3H, s), which was attributed to N—CH_3 in high field. Three signals of labile protons at δ 9.30 (1H, br s), 5.78 (2H, br s), and 5.40 (1H, d, $J = 3.6$ Hz) could be observed, which disappeared after adding D_2O. Besides, typical ABX conjugation system proton signals belonging to the fragment of —CH(OH)—CH_2—N could be observed at δ 4.63 (1H, m), 3.27 (1H, dd, $J = 14.2$, 7.8 Hz), and 3.20 (1H, dd, $J = 14.2$, 4.6 Hz). The ^{13}C NMR and DEPT spectra furnished 10 carbon signals, including a methyl, a methylene, five methenyls and three quaternary carbons. The signal at δ 159.4 due to an urea carbonyl, as well as the signals attributed to a *para*-substituted benzene ring at δ 156.3, 134.1, 127.0, and 114.7, was found in the low field of ^{13}C NMR spectrum. In the ^1H–^1H COSY, HSQC, and DEPT experiments, the carbon and proton signals could be attributed one by one. Long-range correlations between H-1 (δ 3.27 and 3.20) and N—CH_3 (δ 35.8), C=O (δ 159.4), C-1′ (δ 134.1), which constructed the fragments above into a whole structure as shown in Figure 1, could be observed in the HMBC spectrum. The relative configuration of the structure was further confirmed by the NOESY correlations that are shown in Figure 1. Thus, the structure of **1** was elucidated as *N*-[2-hydroxy-2-(4-hydroxyphenyl)ethyl]-*N*-methylurea.

For compound **2**, ESI-MS gave its quasi-molecular ion peak at *m/z* 757 $[M + H]^+$. The extract molecular weight [757.3658, $(M + H)^+$, calcd 757.3655] and the molecular formula ($C_{38}H_{52}N_4O_{12}$) were given by high-resolution ESI-MS measurement. The UV spectrum of **2** showed absorption maximum due to an

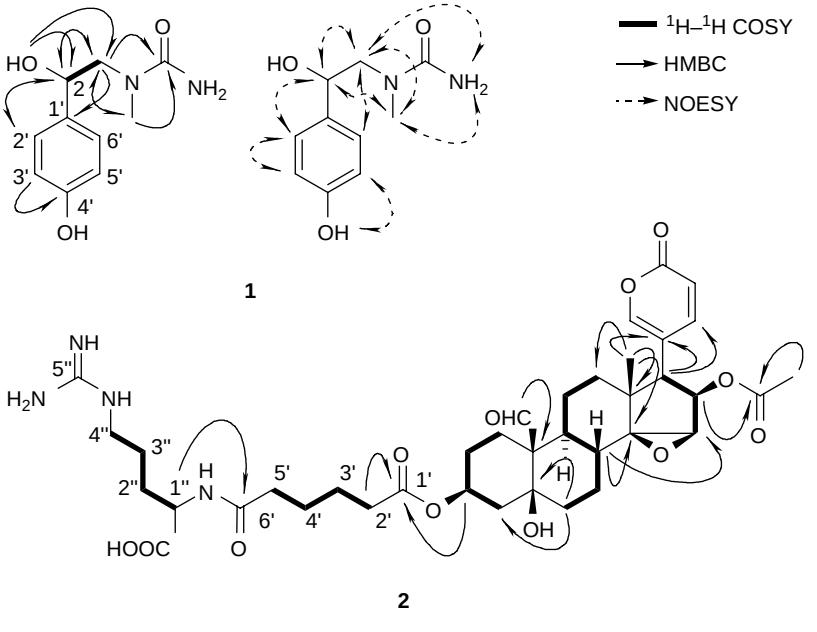

Figure 1. Structures and key ^1H–^1H COSY, HMBC, NOE correlations of compounds **1** and **2**.

α-pyrone group at 293.2 nm. The IR absorption bands at 3428, 2939, 1736, 1639, and 1541 cm^{-1} reveal the existence of hydroxyl, methyl, and α-pyrone groups. The ^{1}H (δ_H 8.18, 7.53, and 6.42) and ^{13}C NMR (δ 161.4, 152.6, 148.8, 116.5, and 113.9) spectral data in low field of **2** also indicated the presence of a *mono*-substituted α-pyrone residue. All the data suggested that compound **2** was a bufadienolide. Signals of two methyls (δ 20.2 and 17.1) and five carbons bearing oxygen (δ 75.2, 72.6, 72.2, 69.6, and 59.8) appeared in the ^{13}C NMR spectrum of **2**. The carbonyl signal (δ 208.3) in the lowest field of the ^{13}C NMR spectrum was attributed to an aldehyde, which showed correlation with the proton singlet (δ 10.49) in HSQC spectrum. The bufadienolide core of **2** was identified as 19-oxocinobufotalin, whose ^{1}H and ^{13}C NMR spectral data were assigned by comparing with those of cinobufotalin (**5**) and further confirmed by the experiment of DEPT and 2D NMR, except for the acylation shifts around C-3 and C-16 of **2**. The arginine structure of **2** was established by comparison of its NMR chemical shifts and proton–proton correlations with those of **12**. Remaining signals (δ 173.1, 29.3, 24.0, 29.2, 34.8, and 173.0) in ^{13}C NMR spectrum belonged to an adipic acid fragment by comparing with corresponding signals in **13**. After acid hydrolysis, the three fragments 19-oxocinobufotalin, arginine, and adipic acid mentioned above were obtained. On the basis of $^{1}H-^{1}H$ COSY, HSQC, and DEPT experiments, the carbon and proton signals can be attributed one by one. Furthermore, long-range correlations between H-1″ (δ 4.86) and C-6′ (δ 173.0), as well as H-3 (δ 5.30) and C-1′ (δ 173.1) in the HMBC spectrum, revealed the linkage of the three fragments. Thus, compound **2** was elucidated as 19-oxocinobufotalin 3-adipoylarginine ester, which had been applied for patent protection for its significant inhibitory activity against the SMMC-7721 cancer cell line [5].

Other known compounds were identified by comparing their ^{1}H and ^{13}C NMR spectral data with those reported in literature. They were characterized as cinobufagin (**3**) [6], bufalin (**4**) [7], cinobufotalin (**5**) [8,9], gamabufotalin (**6**) [10], marinobufagin (**7**) [11], deacetylcinobufagin (**8**) [9], de-*O*-acetylcinobufotalin (**9**) [9], bufotalinin (**10**) [7], bufotoxin (**11**) [12], cinobufotalitoxin (**12**) [9], and cinobufagin 3-adipoylarginine ester (**13**) [13].

Most of the compounds, isolated from the skin of *B. b. gargarizans*, showed significant cytotoxicities against the human hepatocellular carcinoma cell line, SMMC-7721. The results are shown in Table 3.

3. Experimental

3.1 General experimental procedures

Melting points were determined on an XT-4 micromelting point apparatus and are uncorrected. Optical rotations were measured on a Perkin-Elmer digital polarimeter. UV spectra were recorded on a Shimadzu UV-260 spectrometer. IR spectra were determined on a Perkin-Elmer 683 infrared spectrometer in KBr pellets. NMR spectra were taken with TMS as an internal standard on a Bruker Avance 400 FT-NMR spectrometer. HR-ESI-MS were measured on a Bruker FTMS Apex III spectrometer and ESI-MS on a Finnigan LCQ Advantage spectrometer. Column chromatography was performed on silica gel (Marine Chemical Factory, Qingdao, China), Sephadex LH-20 (Amersham Pharmacia Biotech AB, Uppsala, Sweden), Cosmosil 75 C_{18}-OPN (Nakalai Tesque Co. Ltd., Toyko, Japan). TLC was conducted on silica gel GF254 (Marine Chemical Factory, Qingdao, China) and RP-18 F254 (Merck, Darmstadt, Germany) plates. Detection was done by spraying 1% $Ce(SO_4)_2$ on 10% aqueous H_2SO_4, followed by heating. HPLC was performed using an ODS

column (Phenomenex LUNA C_{18} 20 × 250 mm, Los Angeles, America).

3.2 Animal material

The skin of *B. b. gargarizans* was purchased in Shandong Province, China, in April 2005, and identified by Dr Yanyan Zhao, School of Pharmacy, Yantai University. A voucher specimen (20050201-CA) has been deposited at the Medical College, Xiamen University, Xiamen, China.

3.3 Extraction and isolation

The dried skin of *B. b. gargarizans* (20 kg) was finely minced and extracted with 95% EtOH under reflux. Evaporation of the solvent under reduced pressure gave the EtOH extract (660 g). The EtOH extract was partitioned with acetic ether and H_2O. Removal of the solvent under reduced pressure from the acetic ether and H_2O-soluble fractions yielded 450 and 200 g of residues, respectively. The acetic ether soluble fraction (120 g) was subjected to normal-phase silica gel column [1.2 kg, $CHCl_3$–MeOH (100:0 → 0:100, v/v)] to give 24 fractions. Each fraction was subjected to further separation using repeated ODS (eluted successively with H_2O, 30, 50, 70% MeOH and MeOH), silica gel [eluted successively with $CHCl_3$–acetone (20:1 → 15:1 → 10:1 → 5:1 → acetone, v/v)], Sephadex LH-20 (eluted with MeOH) column chromatographies, and preparative HPLC (Phenomenex LUNA C_{18} 20 × 250 mm, CH_3CN–H_2O 45:55) to give eight compounds, identified as cinobufagin (**3**, 878 mg), bufalin (**4**, 124 mg), cinobufotalin (**5**, 42 mg), gamabufotalin (**6**, 56 mg), marinobufagin (**7**, 24 mg), deacetylcinobufagin (**8**, 23 mg), de-*O*-acetylcinobufotalin (**9**, 19 mg), and bufotalinin (**10**, 20 mg). Macroporous resin column (HPD-100) was applied in the separation of water-soluble extract. Three fractions were afforded after eluted gradually with water and alcohol, and they were H_2O eluent, 30% EtOH eluent, and 95% EtOH eluent, respectively. *N*-[2-Hydroxy-2-(4-hydroxyphenyl)ethyl]-*N*-methylurea (**1**, 27 mg) was isolated from the H_2O residue. The residue of 95% EtOH eluent was further separated using repeated ODS (eluted successively with H_2O, 30, 50, 70% MeOH and MeOH) and preparative HPLC (Phenomenex LUNA C_{18} 20 × 250 mm, CH_3CN–H_2O 35:65) to give four bufatoxins, characterized as 19-oxocinobufotalin 3-adipoylarginine ester (**2**, 29 mg), bufotoxin (**11**, 46 mg), cinobufotalitoxin (**12**, 350 mg), and cinobufagin 3-adipoylarginine ester (**13**, 15 mg).

3.3.1 *N-[2-Hydroxy-2-(4-hydroxyphenyl) ethyl]-N-methylurea (**1**)*

White powder; mp 168–170°C; $[\alpha]_D^{25}$ +50.0 ($c = 1.0$, MeOH); UV λ_{max}^{MeOH} nm (log ε) (MeOH): 224.6 (3.81); IR ν_{max} (KBr): 3432, 2925, 2855, 1642, 1542 cm^{-1}; ^1H NMR (DMSO-d_6, 400 MHz); and ^{13}C NMR (DMSO-d_6, 100 MHz) spectral data are shown in Table 1; positive ion ESI-MS: *m/z* 211 $(M + H)^+$; HR-ESI-MS: *m/z* 211.1074 $[M + H]^+$ (calcd for $C_{10}H_{15}N_2O_3$, 211.1077).

3.3.2 *19-Oxocinobufotalin 3-adipoylarginine ester (**2**)*

White powder; mp 184–185°C; $[\alpha]_D^{25}$ +70.0 ($c = 1.0$, MeOH); UV λ_{max}^{MeOH} nm (log ε) (MeOH): 295.2 (3.68); IR ν_{max} (KBr): 3428, 2939,1736, 1639, 1541 cm^{-1}; ^1H NMR (C_5D_5N, 400 MHz); and ^{13}C NMR spectral data (C_5D_5N, 100 MHz) are shown in Table 2; positive ion ESI-MS: *m/z* 757 $(M + H)^+$, HR-ESI-MS: *m/z* 757.3658 $[M + H]^+$ (calcd for $C_{38}H_{53}N_4O_{12}$, 757.3655).

3.3.3 *Acid hydrolysis of compound **2***

Compound **2** (2.0 mg) in 10% HCl–dioxane (1:1, 1 ml) was heated at 80°C

Table 1. ^{1}H and ^{13}C NMR spectral data of compound **1**.

No.	^{1}H NMR	^{13}C NMR
1	3.27 (1H, dd, $J = 14.2$, 7.8 Hz) and 3.20 (1H, dd, $J = 14.2$, 4.6 Hz)	56.8
2	4.63 (1H, m)	71.3
1′		134.1
2′, 6′	7.13 (2H, d, $J = 8.4$ Hz)	127.0
3′, 5′	6.73 (2H, d, $J = 8.4$ Hz)	114.7
4′		156.3
=CO		159.4
N—CH_3	2.70 (3H, s)	35.8
ph—OH	9.30 (1H, br s)	
—NH_2	5.78 (2H, br s)	
2-OH	5.40 (1H, d, $J = 3.6$ Hz)	

for 4 h in a water bath. The reaction mixtures were neutralized with Ag_2CO_3, filtered, and then extracted with $CHCl_3$ (1 ml × 3). After concentration, 19-oxocinobufotalin was found in the $CHCl_3$ layer and the H_2O layer was examined by TLC with *n*-BuOH–AcOH–H_2O (4:1:5, upper layer) as development solvent and compared with arginine and suberic acid.

3.4 MTT assay

Tumor cells (1×10^4 cells/ml) were seeded to RPMI 1640 medium, supplemented with 10% heat-inactivated FBS and kanamin (0.1 mg/ml) at 37°C in a humidified atmosphere containing 5% CO_2 for 24 h. Test compounds, as well as the positive control, taxol, were added to this culture and incubated at 37°C for

Table 2. ^{1}H and ^{13}C NMR spectral data of compound **2**.

No.	^{1}H NMR	^{13}C NMR	No.	^{1}H NMR	^{13}C NMR
1	2.66 (1H, m) and 1.89 (1H, m)	18.8	1′		173.1
2	1.69 (2H, m)	26.1	2′	1.26 (2H, m)	29.3
3	5.30 (1H, br s)	69.6	3′	1.97 (2H, m)	24.0
4	2.37 (1H, m) and 1.93 (1H, m)	36.5	4′	1.26 (2H, m)	29.2
5		72.6	5′	2.32 (1H, m)	34.8
6	2.37 (1H, m) and 1.83 (1H, m)	37.0	6′		173.0
7	1.70 (1H, m) and 1.19 (1H, m)	24.1	1″	4.86 (1H, br s)	54.8
8	2.68 (1H, m)	33.5	2″		30.6
9	1.85 (1H, m)	41.9	3″	1.60 (2H, m)	25.1
10		54.6	4″		41.5
11	1.64 (1H, m) and 1.46 (1H, m)	22.1	5″		158.9
12	1.70 (1H, m) and 1.47 (1H, m)	39.5	CO*CH_3*		20.2
13		45.0	*CO*CH_3		169.9
14		72.2	—COOH		178.1
15	3.95 (1H, br s)	59.8			
16	5.64 (1H, d, $J = 9.3$ Hz)	75.2			
17	3.04 (1H, d, $J = 9.3$ Hz)	50.2			
18	0.82 (3H, s)	17.1			
19	10.49 (1H, s)	208.3			
20		116.5			
21	7.53 (1H, br s)	152.6			
22	8.18 (1H, br d, $J = 9.9$ Hz)	148.8			
23	6.42 (1H, d, $J = 9.9$ Hz)	113.9			
24		161.4			

Table 3. Cytotoxicities of isolated bufadienolides (IC_{50}, $\mu g\,ml^{-1}$).

Compound	**2**	**3**	**4**	**5**	**6**	**7**	**11**	**12**	Taxol
SMMC-7721	2.7	0.38	0.12	0.26	0.0001	4.72	0.27	1.95	0.02

further 120 h without medium change. Cell viability was then evaluated by the MTT-reducing test and compared with that of the control culture, where the cells were treated without adding test compounds.

3.5 Statistics

Values are shown as mean ± SE. Statistical analysis was done by Student's *t*-test and one-way ANOVA, and $p < 0.05$ was considered significant.

3.6 Biological activity

Most of the compounds showed significant cytotoxicities against the SMMC-7721 cancer cell lines. The results are shown in Table 3.

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References

[1] Y. Zhang, Y.K. Qiu, and K. Liu, *Chin. Tradit. Herb. Drugs* **37**, 1905 (2006).
[2] Y. Zhang, Y.K. Qiu, and J.Y. Chen, *J. Shenyang Pharm. Univ.* **24**, 484 (2007).
[3] B. Giri, A. Gomes, and A. Debnath, *Toxicon* **48**, 388 (2006).
[4] H.Y. Zhao, F.K. Wu, Y.K. Qiu, Z. Wu, Y.T. Jiang, and J.Y. Chen, *J. Asian Nat. Prod. Res.* **12**, 793 (2010).
[5] Y.K. Qiu, Y.T. Jiang, J.Y. Chen, J. He, and Z. Li, CN Patent No. 101177445A (14 May 2008).
[6] M. Ye, G.Q. Qiu, H.Z. Guo, and D.A. Guo, *Appl. Environ. Microbiol.* **70**, 3521 (2004).
[7] R. Verpoorte, Q.K. Phan, and S.A. Baerheim, *J. Nat. Prod.* **43**, 347 (1980).
[8] X.W. Yang, Z.T. Xing, J.R. Cui, and Y.L. Fan, *J. Beijing Med. Univ.* **33**, 199 (2001).
[9] M. Ye, L.L. Ning, J.X. Zhan, H.Z. Guo, and D.A. Guo, *J. Mol. Catal. B-Enzym.* **22**, 89 (2003).
[10] Y.J. Chen, J. Xiang, W. Gu, and M.J. Xu, *China J Chin. Mater. Med.* **23**, 620 (1988).
[11] M. Ye, H. Guo, H.Z. Guo, J. Han, and D.A. Guo, *J. Chromatogr. B* **838**, 86 (2006).
[12] R.P. George, K. Yoshiaki, D. Pavel, I. Masuo, and C.K. John, *J. Org. Chem.* **52**, 3573 (1987).
[13] M. Ye, J. Han, H.Z. Guo, and D.A. Guo, *Magn. Reson. Chem.* **40**, 786 (2002).