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Biotransformation of Cinobufagin by *Cunninghamella elegans*

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Abstract *Cunninghamella elegans* has been employed for the biotransformation of cinobufagin to afford 5 metabolites. The structures of the transformation products have been characterized as 12α-hydroxybufagin, 11α-hydroxybufagin, 12β-hydroxy-desacetylcinobufagin, 3-oxo-12α-hydroxybufagin and 12β-hydroxybufagin. Products 12α-hydroxybufagin and 11α-hydroxybufagin are new compounds. *In vitro* both the biotransformation products and cinobufagin all showed cytotoxic activities against HeLa cells.

Keywords biotransformation, cinobufagin, 12α-hydroxybufagin, 11α-hydroxybufagin, *Cunninghamella elegans*

Introduction

Bufadienolides represent a type of steroid with A/B *cis* and C/D *cis* structures and β-2-pyrone ring at the 17-position [1]. As a rule, the oxirane ring is only found in bufadienolides from animal sources, although bufadienolides are also widely distributed in plants such as *Urginea* species [2, 3]. These compounds possess potent cardiotonic, blood pressure-stimulating, antiviral, and local anesthetic activities. Recently, it has been reported that they also had significant inhibitory activities against human myeloid leukemia cells (K562, U937, ML1, HL60), and prostate cancer cells (LNCaP, DU105, PC3) [4~6]. Among those bufadienolides, cinobufagin was most intensively investigated for its abundance in toad venom, but the poor solubility in water restricted its clinical use. Therefore, biotransformation was investigated to modify the structure of cinobufagin to increase the water-solubility to improve its clinical use [7~9]. *Cunninghamella elegans* has been employed for the biotransformation of cinobufagin (**1**) to afford 5 metabolites. The structures of the transformation products have been characterized as 12α-hydroxybufagin (**2**), 11α-hydroxybufagin (**3**), 12β-hydroxy-desacetylcinobufagin (**4**), 3-oxo-12α-hydroxybufagin (**5**) and 12β-hydroxybufagin (**6**) [10] (Fig. 1).

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Experimental

General Experimental Procedures

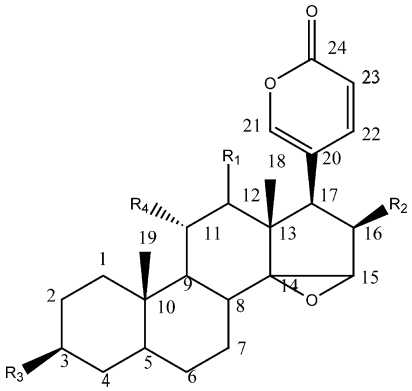
All the NMR spectra were taken on a Bruker-ARX-600 spectrometer (^{1}H at 600 MHz and ^{13}C at 150 MHz). HR-ESI-MS spectra were measured on a MicroMass Autospec-UltimaE TOF spectrometer. Column chromatography was performed on silica gel G (200~300 mesh, Qingdao Haiyang Chemical Factory), and preparative HPLC (Shimadzu LC-8A vp).

Microorganisms and Medium

The *C. elegans* AS 3.156 culture was provided by the Institute of Microbiology, Chinese Academy of Sciences. Microbial cultures were maintained on potato dextrose agar slants at 4°C and transferred every 6 months to maintain viability. The Samaniego medium consisted of glucose 40.0 g, peptone 10.0 g and NaCl 5.0 g. The ingredients were dissolved in 1.0 liter of double distilled deionized water. The pH was adjusted to 6.5 with 5M HCl.

Biotransformation Procedures

Cinobufagin was separated from Chinese drug Chan'Su (obtained in Anguo Folk-Medicinal Market, Hebei



	R_1	R_2	R_3	R_4
1	H	$OCOCH_3$	OH	H
2	α-OH	$OCOCH_3$	OH	H
3	H	$OCOCH_3$	OH	OH
4	β-OH	OH	OH	H
5	α-OH	$OCOCH_3$	=O	H
6	β-OH	$OCOCH_3$	OH	H

Fig. 1 Structures of cinobufagin (**1**), 12α-hydroxybufagin (**2**), 11α-hydroxybufagin (**3**), 12β-hydroxy-desacetylcinobufagin (**4**), 3-oxo-12α-hydroxybufagin (**5**) and 12β-hydroxybufagin (**6**)

province, China, in March of 2005, and identified as dried secretion of *Bufo bufo gargarizans Cantor* by Prof. Qi-shi Sun) and the purity was about 98%. The substrate was dissolved in ethanol as a 10 mg/ml solution. For screening analysis, the microbial strain was incubated in 100-ml conical flasks, each containing 20 ml Samaniego medium. The culture medium was incubated for 48 hours at 220 rpm (29°C). Subsequently, 2 mg of substrate was added as an ethanol solution. Fermentation was allowed to proceed for 5 days. The whole broth was then filtered under vacuum, and the filtrate extracted with ethyl acetate (three times). The extract was then evaporated to give a residue that was dissolved with methanol for TLC analysis using chloroform - acetone - cyclohexane (3 : 3 : 4) as eluting solvent. To prepare the biotransformation products, *C. elegans* was incubated in 24 one-liter conical flasks, each containing 250 ml of Samaniego medium. The culture medium was incubated for 48 hours at 220 rpm (29°C). Cinobufagin (600 mg) was then added to the *C. elegans* cultures.

Extraction and Isolation

About 800 mg of extract was obtained from the incubation of the substrate with *C. elegans*. The extract was chromatographed on a silica gel column eluted with a gradient of chloroform - acetone. Five transformed products, **2** (5 mg), **3** (3 mg), **4** (5 mg), **5** (6 mg) and **6** (20 mg) were obtained (Fig. 1); the approx. yields were 0.83%, 0.5%, 0.83%, 1.0% and 3.3%.

2: White amorphous powder; $C_{26}H_{34}O_7$; mp 223~225°C; $[\alpha]_D^{25}$ −10.6° (*c*, 0.01, MeOH); UV λ_{max} (MeOH) nm 298, 203; IR ν_{max} (KBr) (cm^{-1}) 3400, 2947, 2871, 1703, 1625, 1240, 1157, 1031, 957 cm^{-1}; ^1H and ^{13}C NMR data see Table 1; HR-ESI-MS *m/z*: 459.2378 $[M+H]^+$ (Calcd for $C_{26}H_{34}O_7$, 459.2383).

3: White amorphous powder; $C_{26}H_{34}O_7$; mp 244~246°C; $[\alpha]_D^{25}$ +13.8° (*c*, 0.01, MeOH); UV λ_{max} (MeOH) nm 295, 203; IR ν_{max} (KBr) (cm^{-1}) 3400, 2953, 2869, 1712, 1635, 1243, 1148, 1031, 958 cm^{-1}; ^1H and ^{13}C NMR data see Table 1; HR-ESI-MS *m/z*: 459.2420 $[M+H]^+$ (Calcd for $C_{26}H_{34}O_7$, 459.2383).

Results and Discussion

Incubation of **1** with *C. elegans* yielded five compounds (**2**~**6**, Fig. 1). TLC analyses showed that the Rfs of **2**~**6** (chloroform - acetone - cyclohexane 3 : 3 : 4, 0.3, 0.3, 0.2, 0.35, 0.3, respectively) were all smaller than that of the substrate (chloroform - acetone - cyclohexane 3 : 3 : 4, 0.7), suggesting that they are more polar than the substrate cinobufagin.

4, **5** and **6** were identified as 12β-hydroxy-desacetyl-cinobufagin, 3-oxo-12α-hydroxybufagin and 12β-hydroxy-bufagin, respectively, by comparison with the literature [10] and on the basis of various spectroscopic analyses.

2 was obtained as a white amorphous powder. Its formula was determined to be $C_{26}H_{34}O_7$ by the $[M+H]^+$ ion peak at *m/z* 459.2378 (calcd for $C_{26}H_{35}O_7$ 459.2383) in the HR-ESI-MS. NMR data are shown in Table 1. The key HMBC and NOESY correlations of **2** are shown in Fig. 2. ^{13}C NMR spectrum showed a new carbon signal at δ 73.3. This suggested that **2** was a hydroxylated product. In HMBC spectrum, the cross-peaks between the proton signal at δ 3.29 and C-20 (δ 116.8), C-21 (δ 151.8), C-22 (δ 149.0) and C-23 (δ 112.8) indicated that δ 3.29 was assigned to H-17. The methyl proton signal at δ 0.65 was assigned to H-18 according to its long-range correlation with C-17 (δ 45.6). The proton signal at δ 0.65 also correlated with C-12 (δ 73.3), C-13 (δ 49.6) and C-14 (δ

Table 1 NMR data of **2** and **3** in DMSO-d_6

Position	**2**		**3**	
	δ_C	δ_H (*J* Hz)	δ_C	δ_H (*J* Hz)
1	29.4	1.88, 1.75	29.6	1.72, 1.75
2	27.6	1.74, 1.66	27.5	1.76, 1.62
3	64.6	3.88 (br s)	64.7	3.87 (br s)
4	33.1	1.76, 1.17	32.9	1.77, 1.18
5	35.1	1.59	35.5	1.69
6	25.7	1.51, 1.08	24.9	1.63, 0.91
7	20.1	1.33, 0.96	20.5	1.78, 1.15
8	32.4	1.90	27.7	2.32 (td)
9	30.9	1.89	40.0	1.64
10	34.7		37.8	
11	28.2		65.0	3.99 (s)
12	73.3	3.56 (br s, 5.7)	47.0	2.95 (dd, 4.5, 9.6)
13	49.6		44.3	
14	70.2		72.9	
15	60.5	3.73 (s)	59.6	3.74 (s)
16	76.1	5.38 (d, 4.5)	74.5	5.45 (d, 4.5)
17	45.6	3.29 (d, 4.5)	49.6	2.79 (d, 4.5)
18	17.3	0.65 (s)	19.9	0.93 (s)
19	23.5	0.88 (s)	26.8	1.09 (s)
20	116.8		116.2	
21	151.8	7.39 (d, 2.1)	152.2	7.45 (d, 2.1)
22	149.0	7.83 (dd, 2.1, 9.6)	148.8	7.83 (dd, 2.1, 9.6)
23	112.8	6.22 (d, 9.6)	112.7	6.21 (d, 9.6)
24	160.9		160.9	
1′	169.4		169.4	
2′	20.8	1.77 (s)	20.2	1.79 (s)
3-OH		4.20 (d, 2.0)		4.17 (d, 1.5)
11-OH				4.33 (d, 1.8)
12-OH		5.04 (d, 2.1)		

70.2), which suggested that the hydroxyl group was at C-12.

The relative stereochemistry of 12-OH was determined by the analysis of NOESY spectrum. A NOESY correlation of H-18/H-12 indicated that the proton of H-12 was β-oriented, so 12-OH was α-oriented.

3 was obtained as a white amorphous powder. Its formula was determined to be $C_{26}H_{34}O_7$ by the $[M+H]^+$ ion peak at *m/z* 459.2420 (calcd for $C_{26}H_{35}O_7$ 459.2383) in the HR-ESI-MS, which was identical to **2**. NMR data and the key HMBC and NOESY correlations of **3** are shown in Table 1 and Fig. 2, respectively. Its UV and IR data were similar to **2**. ^1H and ^{13}C spectra of **3** showed a new proton at δ 3.99 and a new carbon signal at δ 65.0, which suggested that **3** was also a hydroxylated product. In HMBC spectrum, the cross-peaks between the proton signal at δ 2.79 and C-20 (δ 116.2), C-21 (δ 152.2), C-22 (δ 148.8) and C-23 (δ 112.7) indicated that δ 2.79 was assigned to H-17. The methyl proton signal at δ 0.93 was assigned to H-18 according to its long-range correlation with C-17 (δ 49.6), C-12 (δ 47.0), C-13 (δ 44.3) and C-14 (δ 72.9). The methyl signal at δ 1.09 with long-range correlations with C-1 (δ 29.6), C-10 (δ 37.8), C-9 (δ 40.0) and C-5 (δ 35.5) was then assigned to H-19. The HMBC correlation between δ 3.99 (the proton signal which correlated with δ 65.0 in HSQC) and C-10, C-13 suggested that the hydroxyl group was at C-11. In the NOESY spectrum, the correlation between H-18/H-11 and H-19/H-11 indicated that H-11 was β-oriented, so 11-OH was α-oriented.

Cytotoxicity Assay

1∼**6** were tested for their cytotoxic activities *in vitro*

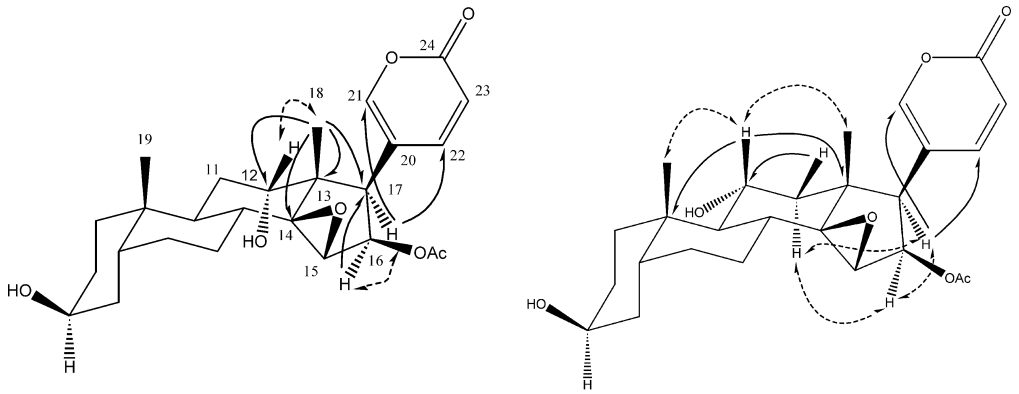


Fig. 2 Key HMBC and NOESY correlations of **2** and **3**. (⟵⟶ NOESY ⟶ HMBC)

Table 2 IC_{50} values of **1**∼**6** (μM) against HeLa cells

Compound	**1**	**2**	**3**	**4**	**5**	**6**
IC_{50} (μM)	0.03	1.85	0.45	0.23	0.78	3.58

against HeLa cells by the MTT method [11]. The IC_{50} values of **1**∼**6** against HeLa cells are given in Table 2. From the table, we can conclude that when the solubility increased, the cytotoxic activity against HeLa cells decreased. That introduction of hydroxy group to C-12 of **1** and deacetylation of C-16 decreased the cytotoxicity, the other hand, introduction of carbonyl group to C-3 increased the cytotoxicity.

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