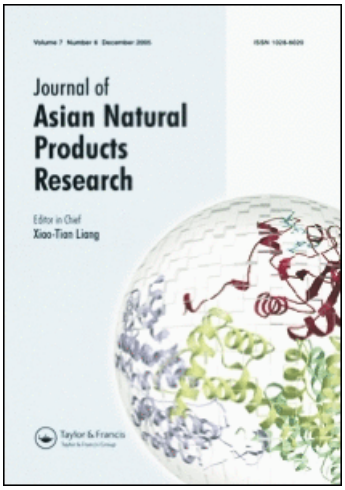

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The microbial transformation of a cytotoxic bufadienolide, bufotalin (**1**), was carried out. Three transformed products from **1** by *Alternaria alternata* were isolated. Their structures were characterized as 3-keto-bufotalin (**2**), 12β-hydroxyl-bufotalin (**3**), and 3-keto-12β-hydroxyl-bufotalin (**4**) based on the extensive NMR studies. Among them, **3** and **4** were new compounds with strong *in vitro* cytotoxic activities against HeLa cells.

Keywords: biotransformation; bufotalin; bufadienolide; *Alternaria alternata*; cytotoxic activity

1. Introduction

The traditional Chinese medicine ChanSu is a product of the skin secretions of local toads [1]. Bufadienolides such as bufalin, bufotalin, cinobufagin, and resibufogenin have steroidal A/B *cis* and C/D *cis* ring junctures with characteristics α-pyrone ring at C-17. They have strong *in vitro* activities against the cancer cells [2–3]. Meanwhile, bufotalin (**1**), as a derivative of bufalin with 16-OAc, had more potent cytotoxicity than bufalin, cinobufagin, and resibufogenin [4]. Microbial transformation is a useful technique to modify the structures of biologically active compounds. More than 80 transformed products from natural bufadienolides with strong cytotoxic activities were obtained by using a biotransformation approach [5–12]. In order to find new chemical entities to improve their activities, the microbial transformation of bufotalin (**1**) by *Alternaria alternata* was investigated. Incubation of **1** with *A. alternata* for 6 days yielded three products (Figure 1), and their structures were identified as 3-keto-bufotalin (**2**), 12β-hydroxyl-bufotalin (**3**), and 3-keto-12β-hydroxyl-bufotalin (**4**), based on the extensive NMR studies including ^{1}H NMR, ^{13}C NMR, DEPT, HMQC, HMBC, and NOESY. Among them, **3** and **4** were new compounds with strong cytotoxic activities against HeLa cells *in vitro.*

2. Results and discussion

The biotransformation of **1** by *A. alternata* was performed and three products were obtained. According to the TLC and HPLC analyses, the transformed products were more polar than the substrate. Site-specific hydroxylation of C-12 and dehydrogenation reaction of C-3 were observed in the biotransformation process of **1**, which was similar to the transformed reactions of bufalin and cinobufagin by *A. alternata* [12].

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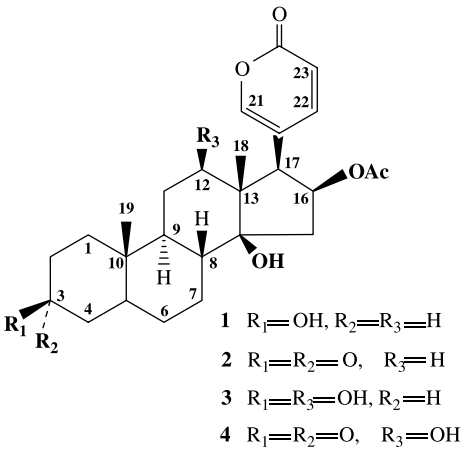

Figure 1. Chemical structures of compounds **1**–**4**.

Compound **3** was obtained as a white powder. It was optically active, with $[\alpha]_D^{22} -45$ (*c* 0.3, MeOH). Its HRFTICRMS provided a pseudo-molecular ion $[M + Na]^+$ at *m/z* 483.2354, suggesting the molecular formula of $C_{26}H_{36}O_7$. Compared with that of compound **1**, the ^{13}C NMR spectrum of compound **3** showed an additional oxygen-bearing tertiary carbon at δ 73.0, indicating that **3** was a monohydroxylated product of **1**. The DEPT spectrum indicated that it had three methyl, seven methylene, 10 methine, and six quaternary carbons. In the HMBC experiment, the carbon signal at δ 73.0 correlated with the proton signals at δ 1.31 (H-11_a), 0.55 (Me-18), and 3.44 (H-17), suggesting that compound **3** possessed a 12-hydroxyl group. In the NOESY spectrum, the proton signal at δ 3.16 (12-H) with the signal at δ 3.44 (17-H) had the NOE enhancement. In contrast to 12α-hydroxylated bufadienolides, where C-18 appeared at remarkably lower fields (δ 18.0), the C-18 of **3** resonated at δ 10.1, due to the *γ-gauche* positions of 12-OH, all of which indicated the β-configuration of the hydroxyl group at C-12. The ^1H and ^{13}C NMR signals of **3** were assigned from its DEPT, COSY, NOESY, HMQC, and HMBC spectral data (Table 1). The key correlations of HMBC and NOESY are shown in Figure 2. On the basis of the above analysis, compound **3** was identified as 12β-hydroxyl-bufotalin.

Compound **4** was obtained as a white powder. It was optically active, with $[\alpha]_D^{22} +34$ (*c* 0.2, MeOH). The molecular formula was established as $C_{26}H_{34}O_7$, according to the pseudo-molecular ion peak at *m/z* 481.2199 $[M + Na]^+$ in the HRFTICRMS spectrum. Compared with that of compound **3**, the ^1H-NMR spectrum of **4** exhibited that the proton signal of H-3 (δ 3.88) disappeared, while the ^{13}C NMR spectrum of **4** showed that the carbon signal of C-3 shifted downfield from δ 64.6 to δ 211.8, suggesting that 3-OH should be changed to a ketone group. The remaining carbon signals of **4** were similar to those of **3**. The DEPT spectrum showed that it had three methyl, seven methylene, nine methine, and seven quaternary carbons. In the HMBC spectrum, the carbon signal at δ 211.8 correlated with the proton signals at δ 1.93 (1-H_a), 2.52 (2-H_a), 2.72 (4-H_a), and 1.74 (5-H), suggesting that compound **4** possessed a 3-ketone group. Moreover, the proton signal at δ 3.26 correlated with the carbon signals at δ 55.4 (13-C) and 83.0 (14-C). The proton signal at δ 0.58 (Me-18) correlated with the carbon signals at δ 55.4 (13-C), 83.0 (14-C), 72.7 (12-C), and 50.9 (17-C), all of which suggested that the additional hydroxyl group was introduced at C-12. Meantime, the carbon signal at δ 169.4 (CO-25) had correlations with the signals at δ 1.78 (Me-26) and 5.30 (16-H), suggesting that 16-OAc was still existing in the molecule of **4**. In the NOESY spectrum, H-12 (δ 3.26) with the signal at δ 3.46 (17-H) had the NOE enhancement, indicating that the hydroxyl group at C-12 should be in the β-configuration. In addition, the configuration of 16-OAc should be β, according to the NOE enhancement between the proton at δ 3.46 (17-H) and the proton at δ 5.30 (16-H). The key correlations of HMBC and NOESY are shown in Figure 3. On the basis of the above analysis, compound **4** was identified as 3-keto-12β-hydroxyl-bufotalin. All the ^1H and ^{13}C NMR spectral data were unambiguously assigned by the 2D NMR spectra.

Table 1. ^{1}H (500 MHz) and ^{13}C (125 MHz) NMR spectral data of **3** and **4** in DMSO-d_6 (δ in ppm, J in Hz).

No.	**3**		**4**	
	δ (H)	δ (C)	δ (H)	δ (C)
1	1.31 (m)	29.5	1.32 (m)	36.2
	1.42 (m)		1.93 (m)	
2	1.29 (m)	27.4	1.99 (m)	36.6
	1.53 (m)		2.52 (m)	
3	3.88 (br s)	64.6	–	211.8
4	1.18 (m)	33.0	1.85 (m)	41.6
	1.80 (m)		2.72 (m)	
5	1.64 (m)	35.6	1.74 (m)	43.1
6	1.11 (m)	26.3	1.22 (m)	26.1
	1.74 (m)		1.73 (m)	
7	1.02 (m)	21.0	1.02 (m)	20.5
	1.70 (m)		1.82 (m)	
8	1.43 (m)	40.8	1.51 (m)	40.7
9	1.56 (m)	31.4	1.83 (m)	31.9
10	–	34.7	–	34.5
11	1.03 (m)	29.4	1.12 (m)	29.5
	1.31 (m)		1.43 (m)	
12	3.16 (m)	73.0	3.26 (m)	72.7
13	–	55.4	–	55.4
14	–	83.0	–	83.0
15	1.66 (m)	39.8	1.66 (m)	40.1
	2.48 (m)		2.48 (m)	
16	5.31 (t, $J = 8.5$)	73.7	5.30 (t, $J = 8.5$)	73.7
17	3.44 (d, $J = 9.0$)	50.9	3.46 (d, $J = 9.0$)	50.9
18	0.55 (s)	10.1	0.58 (s)	10.1
19	0.84 (s)	23.6	0.91 (s)	21.9
20	–	117.4	–	117.4
21	7.41 (br s)	151.3	7.42 (d, $J = 2.0$)	151.2
22	8.13 (dd, $J = 2.5$, 10.0)	150.2	8.13 (dd, $J = 2.0$, 9.5)	150.2
23	6.18 (d, $J = 10.0$)	111.7	6.19 (d, $J = 9.5$)	111.7
24	–	161.0	–	161.1
25	–	169.4	–	169.4
26	1.90 (s)	20.6	1.78 (s)	20.7

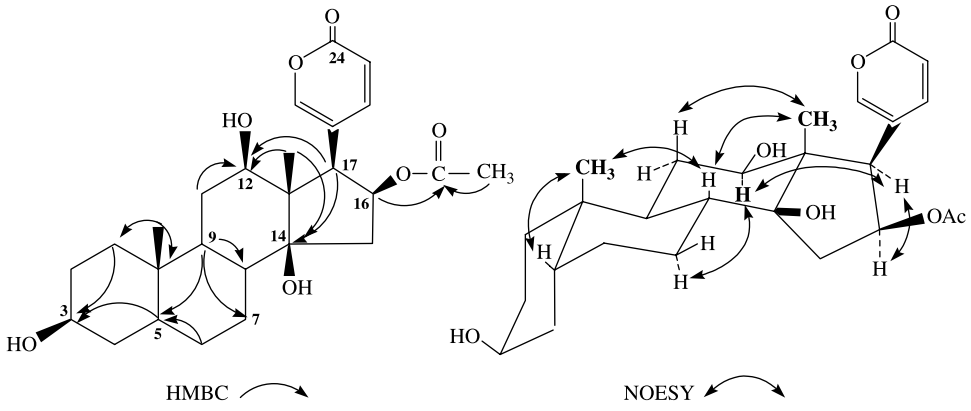

Figure 2. Key HMBC and NOESY correlations of **3**.

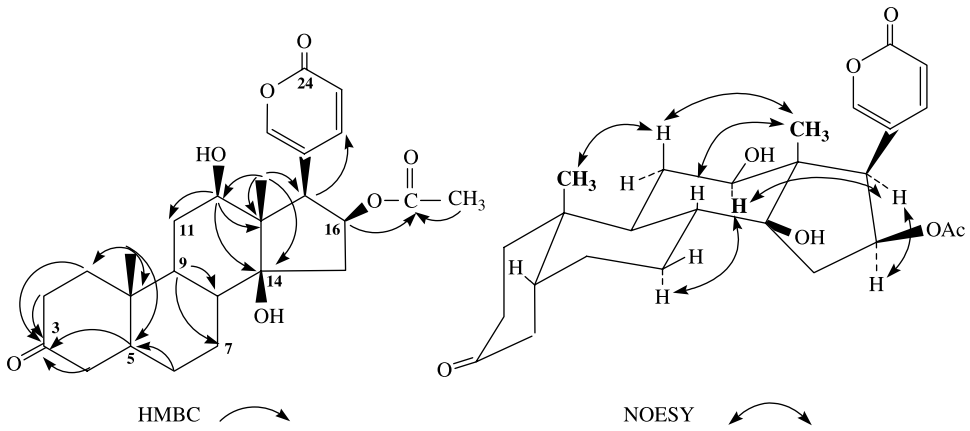

Figure 3. Key HMBC and NOESY correlations of **4**.

The MTT bioassay showed that compounds **1**–**4** had significant *in vitro* cytotoxicities against HeLa (human cervical carcinoma) cells, with IC_{50} values of 3.8×10^{-3} (**1**), 7.93×10^{-2} (**2**), 6.42×10^{-2} (**3**), and 6.53×10^{-2} (**4**) μM. All metabolites had less potent activities than **1**, showing that 3-dehydrogenation or 12β-hydroxylation may lead to the decrease of cytotoxicity.

3. Experimental

3.1 General experimental procedures

Optical rotations were measured with a Perkin–Elmer 243B polarimeter. The UV spectra were detected on a YV-1091 UV–Vis spectrophotometer. The IR spectra were obtained on an Avatar 360 FT-TR spectrophotometer. The NMR spectra were recorded on a Bruker DRX-500 spectrometer (500 MHz for ^{1}H NMR and 125 MHz for ^{13}C NMR) in DMSO-d_6 with TMS as an internal standard. HRMS were obtained on a Bruker APEXII Fourier transform ion cyclotron resonance (FT-ICR) mass spectrometer. Agilent 1100 series HPLC equipped with a diode array detector at 296 nm. Silica gel (200–300 mesh) was purchased from Qingdao Marine Chemical Group, Qingdao, China. Bufotalin (**1**) was isolated from ChanSu by the author. The purity was above 98% determined by HPLC.

3.2 Microorganisms

Mucor spinosus AS 3.3450, *M. spinosus* AS 3.3447, *Mucor subtilissimus* AS 3.2454, *M. subtilissimus* AS 3.2456, *Mucor polymorphosporus* AS 3.3443, *Cunninghamella blakesleana lender* AS 3.970, *Cunninghamella elegans* AS 3.1207, *C. elegans* AS 3.2028, *A. alternata* AS 3.4578, *Alternaria longipes* AS 3.2875, *Penicillium melinii* AS 3.4474, *P. janthinellum* AS 3.510, *Syncephalastrum racemosum* AS 3.264, *Trichoderma viride* AS 3.2942, *Rhizopus stolonifer* AS 3.3463, *R. stolonifer* AS 3.2050, *Rhizopus arrhizus* AS 3.2897, and *Curvularia lunata* AS 3.4381 were purchased from the China General Microbiological Culture Collection Center in Beijing, China.

3.3 Culture medium

All cultures of filamentous fungi were performed in potato medium, which was made of the following composition (L): 200 g potato and 20 g glucose [13].

3.4 Biotransformation procedures

Screening scale biotransformation of bufotalin was carried out in 250 ml Erlenmeyer flasks with 100 ml of medium. The flasks were placed on a rotary shaker operating at 180 rpm at 27°C. The substrates were

dissolved in acetone to reach a concentration of 10 mg/ml. After 36 h of pre-culture, 0.2 ml of its solution was added into each flask and these flasks were maintained under the fermentation conditions for 6 days. Culture controls were used as fermentation blanks in which microorganisms were grown without the substrate but were added with the same amount of acetone. Substrate controls contained the sterile medium with the same amount of substrate and were incubated under the above conditions.

Preparative scale biotransformation of bufotalin by *A. alternata* was carried out in 1000 ml Erlenmeyer flasks. After 36 h of pre-culture, 10 mg of substrates with 1 ml acetone were added into each flask containing 400 ml culture medium. In total, 200 mg of substrates were used to preparative biotransformation. The incubation was continued for six additional days. Other procedures were the same as in the screening scale biotransformation.

3.5 Extraction and isolation

The culture was filtered and the filtrate was extracted with the same volume of EtOAc five times. The organic phase was collected and concentrated *in vacuo*. The residues were applied to a silica gel column and eluted with petroleum ether–acetone in a gradient manner from 100:1 to 1:1, to obtain the products **2** (8 mg), **3** (7 mg), and **4** (8 mg), respectively.

12β-Hydroxyl-bufotalin (**3**): colorless needles (MeOH), showing a pink spot with 10% H_2SO_4. $[\alpha]_D^{22}$ −45 (*c* 0.3, MeOH), UV λ_{max} MeOH (nm): 298. IR (KBr) ν_{max} (cm^{-1}): 3438, 2936, 1720, 1630, 1248, 1029, and 754. ^1H and ^{13}C NMR spectral data (Table 1). HRFTICRMS: *m/z* 483.2354 $[M + Na]^+$ (calcd for $C_{26}H_{36}O_7Na$, 483.2353).

3-Keto-12β-hydroxyl-bufotalin (**4**): colorless needles (MeOH), showing a pink spot with 10% H_2SO_4. $[\alpha]_D^{22}$ +34 (*c* 0.2, MeOH), UV λ_{max} MeOH (nm): 298. IR (KBr) ν_{max} (cm^{-1}): 3433, 2943, 1715, 1633, 1248, and 1023. ^1H and ^{13}C NMR spectral data (Table 1). HRFTICRMS: *m/z* 481.2199 $[M + Na]^+$ (calcd for $C_{26}H_{34}O_7Na$, 481.2197).

3.6 Bioassay

Human cervical carcinoma (HeLa) cells were maintained in RPMI-1640 medium (GIBCO/BRL, Maryland, USA) supplemented with 10% (v/v) fetal bovine serum and culture in 96-well microtiter plates for the assay. Appropriate dilutions of the test compounds were added to the cultures. Subsequently, the cells were cultured at 37°C, 5% CO_2 for 72 h. The survival rates of the cancer cells were evaluated by the MTT method. The activity was shown as the IC_{50} value, which is the concentration of the test compound to give 50% inhibition of cell growth. The results were expressed as the mean value of triplicate determinations.

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