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Novel cytotoxic bufadienolides derived from bufalin by microbial hydroxylation and their structure–activity relationships

Min Ye, Guiqin Qu, Hongzhu Guo, Dean Guo*

The State Key Laboratory of Natural and Biomimetic Drugs, School of Pharmaceutical Sciences, Peking University, Xueyuan Road #38, Beijing 100083, PR China

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Abstract

Microbial transformation was used to prepare novel cytotoxic bufadienolides. Twelve products (**3**–**14**) were obtained from bufalin (**1**) by the fungus *Mucor spinosus*. Their structures were elucidated by high-resolution mass spectroscopy (HR-MS) and extensive NMR techniques, including ^{1}H NMR, ^{13}C NMR, DEPT, ^{1}H–^{1}H correlation spectroscopy (COSY), two dimensional nuclear Overhauser effect correlation spectroscopy (NOESY), heteronuclear multiple quantum coherence (HMQC), and heteronuclear multiple bond coherence (HMBC). Compounds **3**, **4**, **9** and **11**–**14** are new mono- or dihydroxylated derivatives of bufalin with novel oxyfunctionalities at C-1β, C-7β, C-11β, C-12β and C-16α positions. The in vitro cytotoxic activities against human cancer cell lines of **3**–**14**, together with 16 biotransformed products derived from cinobufagin (**15**–**30**) were determined by the MTT method, and their structure–activity relationships (SAR) were discussed.

Keywords: Bufalin; Bufadienolide; Microbial transformation; *Mucor spinosus*; Cytotoxicity; Structure–activity relationship

1. Introduction

Bufadienolides are a new type of natural steroids with potent antitumor activities, originally isolated from the traditional Chinese drug Chan'Su [1,2]. They have been reported to exhibit significant inhibitory activities against human myeloid leukemia cells (K562, U937, ML1, HL60), human hepatoma cells (SMMC7221), and prostate cancer cells (LNCaP, DU105, PC3). The activities are mediated by induction of cell apoptosis and cell differentiation, and the regulations of a variety of genes and proteins are involved in the process [3–8]. Bufalin (**1**) and cinobufagin (**2**) are two major components of Chan'Su (Fig. 1), and their contents in the crude drug could be as high as 1–5% of the dry weight [9]. Bufalin also is one of the most potent bufadienolides against cancer cells, with IC_{50} values of 10^{-9} to 10^{-10} mol/l.

Recently, Kamano et al. [10,11] obtained 80 bufadienolides and studied their structure–activity relationships (SAR) and QSAR on the inhibition of colchicines-resistant primary liver carcinoma PLC/PRF/5 cells. It was found that subtle changes in functionality of bufadienolides could significantly alter their cytotoxic activities. The essential structural requirements for increasing the inhibitory activities have been identified. Unfortunately, all the test bufadienolides are natural products isolated from Chan'Su, or their chemical derivatives, and the oxyfunctionality sites are restricted to C-3, C-5, C-15 and C-16β positions. The cytotoxicities of bufadienolides oxygenated at other sites, which are obviously difficult to obtain by chemical means, still remain unknown.

Biotransformation, however, is an alternative tool in the structural modification of complex natural products due to its great capabilities to catalyze novel reactions and its regio- and stereo-selectivity [12,13]. Microorganisms, especially filamentous fungi, are well known as efficient and selective hydroxylation catalysts [14–16]. In previous papers we reported the biotransformations of cinobufagin by plant cell suspension cultures and obtained a series of new glucosylated products [17]. The present study intends to produce new bufadienolides from bufalin by microbial transformation. The cytotoxicities of the obtained products were also described.

* Corresponding author. Tel.: +86 10 82801516; fax: +86 10 82802700.
E-mail address: gda@bjmu.edu.cn (D. Guo).

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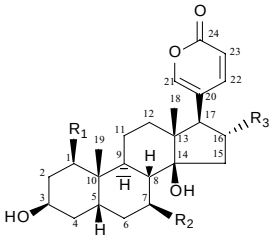
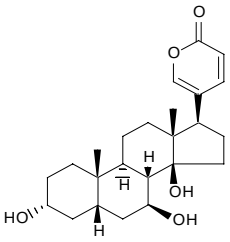
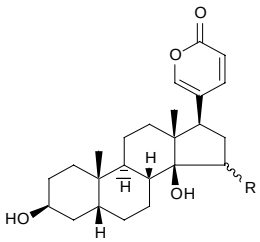


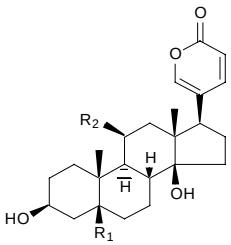
1 bufalin
$R_1=R_2=R_3=H$
3 7β-hydroxyl bufalin
$R_1=R_3=H$ $R_2=OH$
5 1β-hydroxyl bufalin
$R_1=OH$ $R_2=R_3=H$
11 1β,7β-dihydroxyl bufalin
$R_1=R_2=OH$ $R_3=H$
12 16α-hydroxyl bufalin
$R_1=R_2=H$ $R_3=OH$
13 7β,16α-dihydroxyl bufalin
$R_1=H$ $R_2=R_3=OH$



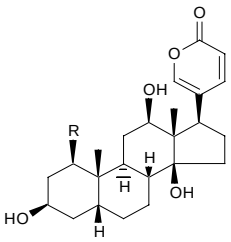
4 3-*epi*-7β-hydroxyl bufalin



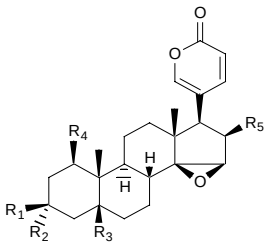
6 15α-hydroxyl bufalin
R=α-OH
7 15β-hydroxyl bufalin
R=β-OH



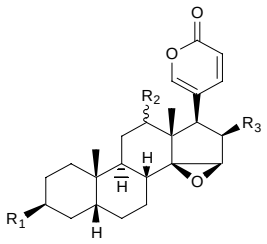
8 telocinobufagin
$R_1=OH$ $R_2=H$
9 11β-hydroxyl bufalin
$R_1=H$ $R_2=OH$



10 12β-hydroxyl bufalin
R=H
14 1β,12β-dihydroxyl bufalin
R=OH



2 cinobufagin
$R_1=OH$ $R_2=R_3=R_4=OH$ $R_5=OAc$
15 desacetylcinobufotalin
$R_1=R_3=R_5=OH$ $R_2=R_4=H$
16 3-*epi*-desacetylcinobufagin
$R_1=R_3=R_4=H$ $R_2=R_5=OH$
17 1β-hydroxyl desacetylcinobufagin
$R_1=R_4=R_5=OH$ $R_2=R_3=H$
18 3-*epi*-desacetylcinobufotalin
$R_1=R_4=H$ $R_2=R_3=R_5=OH$
19 cinobufagin 3-O-β-D-glucoside
R_1=O-glc $R_2=R_3=R_4=H$ $R_5=OAc$
20 3-*oxo*-desacetylcinobufagin 16-O-β-D-glucoside
$R_1+R_2=O$ $R_3=R_4=H$ R_5=O-glc
21 desacetylcinobufagin 16-O-β-D-glucoside
$R_1=OH$ $R_2=R_3=R_4=H$ R_5=O-glc
22 3-*epi*-desacetylcinobufagin 16-O-β-D-glucoside
$R_1=R_3=R_4=H$ $R_2=OH$ R_5=O-glc
23 cinobufotalin
$R_1=R_3=OH$ $R_2=R_4=H$ $R_5=OAc$
24 desacetylcinobufagin
$R_1=R_5=OH$ $R_2=R_3=R_4=H$



25 12β-hydroxyl cinobufagin
$R_1=OH$ R_2=β-OH $R_3=OAc$
26 12β-hydroxyl desacetylcinobufagin
$R_1=R_3=OH$ R_2=β-OH
27 3-*oxo*-12β-hydroxyl cinobufagin
R_1=O R_2=β-OH $R_3=OAc$
28 3-*oxo*-12β-hydroxyl desacetylcinobufagin
R_1=O R_2=β-OH $R_3=OH$
29 12-*oxo*-cinobufagin
$R_1=OH$ R_2=O $R_3=OAc$
30 3-*oxo*-12α-hydroxyl cinobufagin
R_1=O R_2=α-OH $R_3=OAc$

Fig. 1. Chemical structures of **1**–**30**.

2. Materials and methods

2.1. General

Melting points were determined with an XT4A apparatus and are uncorrected. Optical rotations were measured with a Perkin-Elmer 243B polarimeter. UV spectra were detected with a TU-1901 UV-Vis spectrophotometer. IR spectra were recorded in KBr with an Avatar 360 FT-IR spectrophotometer. ^{1}H and ^{13}C 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 at ambient temperature with tetramethylsilane (TMS) as the internal standard. The chemical shifts (δ values) are given in parts per million (ppm) relative to TMS at 0 ppm. The coupling constants (J values) are reported in hertz (Hz). Standard pulse sequences were used for DEPT (distortionless enhancement by polarization transfer), ^{1}H–^{1}H correlation spectroscopy (COSY), two dimensional nuclear Overhauser effect correlation spectroscopy (NOESY), heteronuclear multiple quantum coherence (HMQC), and heteronuclear multiple bond coherence (HMBC) experiments. Matrix-assisted laser desorption ionization time-of-flight (MALDI-TOF) mass spectra were measured on a Bruker BIFLEX III mass spectrometer. High-resolution mass spectra (HR-MS) were obtained on a Bruker APEX II Fourier transform ion cyclotron resonance (FT-ICR) mass spectrometer in the positive mode with a secondary ion mass spectrometry (SIMS) ion source.

2.2. Chemicals

Bufalin (**1**) was isolated from the Chinese drug Chan'Su, and unambiguously identified by NMR and MS techniques. The purity was determined to be >99.5% by HPLC analysis. Silica gel (200–300 mesh) for column chromatography was purchased from Qingdao Marine Chemical Corporation, Qingdao, China. Sephadex LH-20 was from Pharmacia. All chemical solvents used for products isolation were of analytical grade or higher.

2.3. Microorganisms and culture media

The microorganisms were purchased from China General Microbiological Culture Collection Center (CGMCC, Beijing, China). The filamentous fungi (20 strains from 9 genera) included *Absidia coerulea* AS 3.3389, *Alternaria* (*A. alternata* AS 3.4578, *A. alternata* AS 3.577, *A. longipes* AS 3.2875), *Aspergillus* (*A. avenaceus* AS 3.4454, *A. flavus* AS 3.3554, *A. niger* AS 3.1858, *A. niger* AS 3.739, *A. niger* AS 3.795), *Cunninghamella* (*C. blakesleana* AS 3.970, *C. elegans* AS 3.1207), *Fusarium avenaceum* AS 3.4594, *Mucor* (*M. polymorphosporus* AS 3.3443, *M. spinosus* AS 3.2450, *M. spinosus* AS 3.3450, *M. subtilissimus* AS 3.2454), *Penicillium* (*P. janthinellum* AS 3.510, *P. melinii* AS 3.4474), *Sporotrichum* sp. AS 3.2882, and *Syncephalastrum racemosum* AS 3.264. The bacteria screened were *Curtobacterium pusillum* AS 1.1905, *Flavobacterium oryzae* AS 1.1584, *Proteus vulgaris* AS 1.1208, and *Pseudomonas aeruginosa* AS 1.860. Strains were maintained on potato agar slants at 4 °C. Fermentations of fungi were carried out in a potato medium [18] consisting of 20 g of potato extract, 20 g of glucose, and 1000 ml of distilled H_2O. Luria-Bertani (LB) medium [19] was used for the fermentation of bacteria. It is composed of 10 g of tryptone (Oxoid Ltd, England), 5 g of yeast extract (Oxoid), 5 g of NaCl, diluted with distilled water to 1000 ml, and adjusted to pH 7.2 with 5% NaOH. The media were sterilized at 121 °C and 1.06 kg/cm^2 for 30 min.

2.4. Preliminary screening tests

The fungal mycelia or bacterial colonies from the agar slants were aseptically transferred to liquid medium. Cultures were incubated in 250-ml Erlenmeyer flasks containing 80 ml of medium at 25 °C with rotary shaking at 180 rpm in the dark. After 48 h incubation, 3 mg of bufalin (**1**, 10 mg/ml in ethanol) was added to the cultures. The incubation continued for 3 days. Then the cultures were filtered through Whatman no. 1 filter paper in vacuo, and the filtrate extracted with an equal volume of ethyl acetate. The organic extract was evaporated to dryness in a rotary evaporator under reduced pressure at 60 °C, and the obtained residue dissolved in 1 ml of methanol for analysis. Thin-layer chromatography (TLC) analyses were carried out on precoated silica gel GF_{254} plates (Qingdao Marine Chemical Corporation, China). The plates were developed with petroleum ether–acetone (1:1, v/v) and visualized by spraying with 10% H_2SO_4 (in ethanol) and heated at 120 °C for 10 min to produce colorful spots. Culture controls consisted of fermentation blanks in which the microorganisms were incubated under identical conditions with no bufalin. Substrate controls were composed of sterile medium to which bufalin was added and were incubated without the microorganisms.

2.5. HPLC analysis

An Agilent 1100 HPLC apparatus equipped with a diode-array detector (DAD) and a quaternary pump system was used. The column was Zorbax Extend-C_{18} (Agilent, USA), 5 μm, ∅ 4.6 mm × 250 mm. The mobile phase was methanol–water (52:48, v/v) for the first 6 min, then linearly gradient to methanol–water (72:28, v/v) over 19 min, and held for 10 min. The flow rate was 1.0 ml/min; detection wavelength was 296 nm; column temperature was 25 °C. The samples were filtered through 0.45-μm filter membranes prior to use. An aliquot of 10 μl of the sample was injected into the apparatus for each analysis.

2.6. Preparative HPLC conditions

For the isolation of biotransformation products, a SepectraSERIES HPLC apparatus (Thermo Quest) with a 100-μl loop was used. The column was PEGASIL ODS packing

(Senshu Pak, Japan), 5 μm, ∅ 10 mm × 250 mm. The flow rate was 2.0 ml/min, and the detection wavelength was 296 nm. The samples were eluted with mixtures of methanol and water.

2.7. Preparative-scale biotransformation of bufalin by *Mucor spinosus* AS 3.3450

Mycelia of *M. spinosus* from agar slants were aseptically transferred to 250-ml Erlenmeyer flasks containing 80 ml of liquid potato medium. The fungus was incubated at 25 °C on a rotary shaker (180 rpm) in the dark for 24 h to make a stock inoculum. Then an amount of 5 ml of the stock inoculum was added to each of the 40 1-l flasks containing 350 ml of potato medium. After 36 h incubation, a total amount of 800 mg of bufalin dissolved in 40 ml of ethanol was distributed equally among the 40 flasks. The incubation was allowed to continue for additional 4 days on the shaker. The cultures were then pooled and filtered through Whatman no. 1 filter paper in vacuo. The filtrate was extracted with 10 l of ethyl acetate for three times. The organic extract was concentrated and evaporated to dryness in a rotary evaporator under reduced pressure at 60 °C to yield 2.47 g of a brownish solid.

2.8. Isolation and purification of biotransformation products

The extract was subjected to silica gel column chromatography (135 g, 200–300 mesh, ∅ 3 cm × 45 cm), and eluted in 100 ml fractions with petroleum ether (60–90 °C) and acetone (4:1 to 1:1, v/v), gradually increasing the proportion of acetone. The fractions were combined under the guidance of TLC analysis. Fr. 11–13 were crystallized in acetone to give **3** (127.6 mg, 16.0% yield). Fr. 17–23 were subjected to preparative RP-HPLC and eluted with methanol-water (60:40, v/v) to give **4** (4.6 mg, 0.6% yield), **5** (16.0 mg, 2.0% yield), **6** (3.0 mg, 0.4% yield), **7** (7.9 mg, 1.0% yield), **8** (8.1 mg, 1.0% yield), and **9** (10.0 mg, 1.2% yield). Fr. 24–37 were subjected to preparative RP-HPLC and eluted with methanol–water (54:46, v/v) to give **10** (88.3 mg, 11.0% yield) and **11** (12.0 mg, 1.5% yield). Fr. 38–55 were crystallized in acetone to give **12** (44.0 mg, 5.5% yield). Fr. 56–64 were purified over a Sephadex LH-20 column and eluted with methanol to give **13** (55.6 mg, 7.0% yield). Fr. 65–69 were purified by preparative RP-HPLC to obtain **14** (3.7 mg, 0.5% yield).

2.8.1. 7β-Hydroxyl bufalin (3)

Colorless needles from acetone; $C_{24}H_{34}O_5$; mp 247–248 °C (decomp.); $[\alpha]_D^{25}$ −1.6° (*c* 0.64, MeOH); UV λ_{max} (log ε) (MeOH): 208.0 (3.68), 300.0 (3.51) nm; IR ν_{max} (KBr): 3367, 2933, 2894, 2865, 1701, 1634, 1544, 1032, 949, 843 cm^{-1}; HR-FT-ICRMS *m/z* calcd for $C_{24}H_{35}O_5$ $[M + H]^+$, 403.2478, found 403.2479; ^1H and ^{13}C NMR data, see Tables 1 and 2.

2.8.2. 3-epi-7β-Hydroxyl bufalin (4)

White powder; $C_{24}H_{34}O_5$; mp 210–214 °C; $[\alpha]_D^{25}$ +16.7° (*c* 0.06, MeOH); UV λ_{max} (log ε) (MeOH): 206.0 (4.08), 298.0 (3.88) nm; IR ν_{max} (KBr): 3371, 2931, 2863, 1707, 1540, 1261, 1143, 1053, 946, 848, 804 cm^{-1}; HR-FT-ICRMS *m/z* calcd for $C_{24}H_{35}O_5$ $[M + H]^+$, 403.2478, found 403.2476; ^1H and ^{13}C NMR data, see Tables 1 and 2.

2.8.3. 1β-Hydroxyl bufalin (5)

White powder; $C_{24}H_{34}O_5$; mp 196–197 °C; $[\alpha]_D^{25}$ −30.0° (*c* 0.33, MeOH); UV λ_{max} (log ε) (MeOH): 210.0 (3.65), 300.0 (3.55) nm; TOF-MS (*m/z*): 403 $[M + H]^+$; ^1H NMR (DMSO-d_6, 500 MHz): 7.91 (1H, d, J = 10.0 Hz, H-22), 7.50 (1H, s, H-21), 6.27 (1H, d, J = 10.0 Hz, H-23), 5.13 (1H, d, J = 4.5 Hz, 3-OH), 4.77 (1H, d, J = 7.5 Hz, 1-OH), 4.14 (1H, s, 14-OH), 3.99 (1H, brs, H-3), 3.58 (1H, brs, H-1), 2.41 (1H, m, H-17), 0.94 (3H, s, 19-CH_3), 0.58 (3H, s, 18-CH_3); ^{13}C NMR data, see Table 1.

2.8.4. 15α-Hydroxyl bufalin (6)

White powder; $C_{24}H_{34}O_5$; mp 236–238 °C; $[\alpha]_D^{25}$ −2.9° (*c* 0.34, MeOH); UV λ_{max} (log ε) (MeOH): 206.0 (3.38), 301.0 (2.99) nm; HR-FT-ICRMS *m/z* calcd for $C_{24}H_{35}O_5$ $[M + H]^+$, 403.2478, found 403.2481; ^1H and ^{13}C NMR data, see Tables 1 and 3.

2.8.5. 15β-Hydroxyl bufalin (7)

White powder; $C_{24}H_{34}O_5$; mp 236–238 °C; $[\alpha]_D^{25}$ −30.0° (*c* 0.30, MeOH); UV λ_{max} (log ε) (MeOH): 208.0 (3.61), 299.0 (3.29) nm; TOF-MS (*m/z*): 403 $[M + H]^+$; ^1H and ^{13}C NMR data, see Tables 1 and 3.

2.8.6. Telocinobufagin (5-hydroxyl bufalin, 8)

White powder; $C_{24}H_{34}O_5$; mp 114–116 °C; $[\alpha]_D^{25}$ −12.6° (*c* 0.16, MeOH); UV λ_{max} (log ε) (MeOH): 206.0 (3.75), 300.0 (3.52) nm; TOF-MS (*m/z*): 403 $[M + H]^+$; ^1H NMR (DMSO-d_6, 500 MHz):7.91 (1H, dd, J = 10.0, 2.0 Hz, H-22), 7.51 (1H, d, J = 2.0 Hz, H-23), 6.27 (1H, d, J = 10.0 Hz, H-21), 5.17 (1H, d, J = 4.0 Hz, 3-OH), 4.77 (1H, s, 5-OH), 4.14 (1H, s, 15-OH), 3.99 (1H, brs, H-3), 2.45 (1H, dd, J = 9.5, 6.0 Hz, H-17), 0.81 (3H, s, 19-CH_3), 0.58 (3H, s, 18-CH_3); ^{13}C NMR data, see Table 1.

2.8.7. 11β-Hydroxyl bufalin (9)

White powder; $C_{24}H_{34}O_5$; mp 213–214 °C; $[\alpha]_D^{25}$ −1.4° (*c* 0.73, MeOH); UV λ_{max} (log ε) (MeOH): 215.0 (3.82), 299.0 (3.62) nm; IR ν_{max} (KBr): 3430, 3382, 2920, 2851, 1712, 1625, 1536, 1060, 1031, 948, 828 cm^{-1}; HR-FT-ICRMS *m/z* calcd for $C_{24}H_{35}O_5$ $[M + H]^+$, 403.2478, found 403.2475; ^1H and ^{13}C NMR data, see Tables 1 and 3.

2.8.8. 12β-Hydroxyl bufalin (10)

White crystalline powder; $C_{24}H_{34}O_5$; mp 228–229 °C; $[\alpha]_D^{25}$ −5.9° (*c* 1.19, MeOH); UV λ_{max} (log ε) (MeOH):

Table 1
^{13}C NMR spectral data of compounds **1** and **3**–**14** (DMSO-d_6, 125 MHz)

C	**1**[a]	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**	**13**	**14**
1	29.5t	29.2t	30.2t	72.0d	29.7t	29.5t	24.9t	29.6t	29.6t	71.9d	29.5t	29.2t	72.1d
2	27.5t	27.3t	33.2t	32.0t	27.7t	27.6t	27.3t	27.8t	27.5t	31.8t	27.6t	27.3t	32.1t
3	64.6d	64.3d	69.6d	66.7d	64.7d	64.6d	66.5d	64.6d	64.5d	66.5d	64.5d	64.3d	66.8d
4	33.1t	33.2t	37.2t	33.0t	33.4t	33.1t	36.5t	33.1t	33.0t	34.0t	33.0t	34.1t	32.9t
5	35.6d	36.4d	41.7d	30.0d	35.9d	35.8d	73.5s	35.9d	35.7d	31.1d	35.6d	36.4d	30.1d
6	26.5t	36.7t	37.0t	26.0t	26.9t	26.6t	34.9t	25.7t	26.4t	36.1t	26.4t	36.6t	25.9t
7	21.1t	69.2d	69.1d	20.9t	20.6t	21.0t	23.3t	21.4t	21.3t	69.1d	21.1t	69.1d	21.0t
8	41.2d	45.9d	46.1d	41.2d	41.8d	41.3d	40.2d	37.5d	40.7d	46.2d	41.0d	45.8d	40.8d
9	34.8d	34.1d	34.9d	36.6d	33.5d	34.4d	38.1d	37.8d	31.5d	36.1d	34.9d	34.1d	33.2d
10	34.9s	34.7s	34.2s	40.0s	35.2s	35.0s	40.3s	35.3s	34.8s	40.0s	34.9s	34.7s	39.8s
11	21.1t	21.2t	20.9t	20.8t	20.3t	19.7t	21.5t	65.3d	29.8t	20.9t	21.1t	21.2t	29.7t
12	40.0t	39.8t	39.8t	39.8t	40.4t	38.9t	40.0t	48.1t	74.0d	39.6t	40.8t	40.6t	73.8d
13	48.0s	47.4s	47.4s	47.8s	47.1s	47.0s	47.9s	47.7s	54.2s	47.2s	47.2s	46.6s	54.0s
14	83.4s	84.4s	84.4s	83.2s	83.5s	80.6s	83.4s	84.4s	84.0s	84.4s	82.9s	83.8s	83.8s
15	32.0t	34.1t	34.5t	31.9t	78.2d	71.9d	31.9t	32.1t	32.2t	33.2t	40.8t	42.1t	32.0t
16	28.4t	28.4t	28.4t	28.4t	39.1t	38.2t	28.4t	28.3t	28.6t	28.4t	76.6d	76.6d	28.6t
17	50.1d	50.0d	50.5d	50.0d	49.2d	47.4d	50.0d	50.9d	45.4d	50.0d	60.3d	60.2d	45.3d
18	16.7q	16.8q	16.8q	16.6q	18.2q	17.1q	16.7q	19.6q	10.2q	16.8q	16.6q	16.7q	10.2q
19	23.7q	23.6q	23.0q	18.7q	22.7q	23.9q	16.6q	27.1q	23.7q	18.6q	23.7q	23.6q	18.7q
20	122.7s	122.6s	122.6s	122.7s	122.1s	122.2s	122.7s	122.8s	122.9s	122.5s	121.2s	121.0s	122.8s
21	149.1d	149.1d	149.1d	149.1d	148.8d	149.5d	149.2d	149.0d	149.2d	149.1d	149.5d	149.6d	149.2d
22	147.4d	147.5d	147.5d	147.3d	147.0d	147.3d	147.3d	147.5d	147.6d	147.5d	147.2d	147.3d	147.5d
23	114.1d	114.2d	114.2d	114.1d	114.3d	114.0d	114.1d	114.0d	114.1d	114.2d	114.3d	114.3d	114.1d
24	161.3s	161.3s	161.3s	161.3s	161.2s	161.2s	161.3s	161.3s	161.3s	161.3s	161.3s	161.3s	161.3s

[a] 75 MHz.

207.0 (3.63), 301.0 (3.38) nm; TOF-MS (*m*/*z*): 403.1 $[M + H]^+$; ^{1}H and ^{13}C NMR data, see Tables 1 and 3.

2.8.9. 1β,7β-Dihydroxyl bufalin (**11**)

White crystalline powder; $C_{24}H_{34}O_6$; mp 250–252 °C (decomp.); $[\alpha]_D^{25}$ −17.5° (*c* 0.34, MeOH); UV λ_{max} (log ε) (MeOH): 210.0 (3.67), 298.0 (3.57) nm; IR ν_{max} (KBr): 3394, 2940, 1719, 1632, 1539, 1447, 1050, 943, 832 cm^{-1}; HR-FT-ICRMS *m*/*z* calcd for $C_{24}H_{35}O_6$ $[M + H]^+$, 419.2428, found 419.2432; ^{1}H and ^{13}C NMR data, see Tables 1 and 2.

2.8.10. 16α-Hydroxyl bufalin (**12**)

Colorless needles from acetone; $C_{24}H_{34}O_5$; mp 189–191 °C; $[\alpha]_D^{25}$ −43.6° (*c* 0.28, MeOH); UV λ_{max} (log ε) (MeOH): 211.0 (3.68), 299.0 (3.47) nm; IR ν_{max} (KBr): 3428, 2936, 2869, 1729, 1633, 1539, 1137, 1040, 948, 831 cm^{-1}; HR-FT-ICRMS *m*/*z* calcd for $C_{24}H_{35}O_5$ $[M + H]^+$, 403.2478, found 403.2479; ^{1}H and ^{13}C NMR data, see Tables 1 and 2.

2.8.11. 7β,16α-Dihydroxyl bufalin (**13**)

Colorless prisms from acetone–methanol; $C_{24}H_{34}O_6$; mp 293–295 °C (decomp.); $[\alpha]_D^{25}$ −21.9° (*c* 1.23, MeOH); UV λ_{max} (log ε) (MeOH): 215.0 (3.63), 299.0 (3.48) nm; IR ν_{max} (KBr): 3356, 2944, 2921, 2879, 1701, 1633, 1542, 1141, 1052, 1033, 964, 835 cm^{-1}; HR-FT-ICRMS *m*/*z* calcd for $C_{24}H_{35}O_6$ $[M + H]^+$, 419.2428, found 419.2429; ^{1}H and ^{13}C NMR data, see Tables 1 and 2.

2.8.12. 1β,12β-Dihydroxyl bufalin (**14**)

Colorless needles from acetone; $C_{24}H_{34}O_6$; mp 268–270 °C; $[\alpha]_D^{25}$ +15.6° (*c* 0.32, MeOH); UV λ_{max} (log ε) (MeOH): 208.0 (3.84), 299.0 (3.67) nm; IR ν_{max} (KBr): 3374, 2937, 1696, 1629, 1538, 1409, 1236, 1135, 1018, 945, 827, 572 cm^{-1}; HR-FT-ICRMS *m*/*z* calcd for $C_{24}H_{35}O_6$ $[M + H]^+$, 419.2428, found 419.2434; ^{1}H and ^{13}C NMR data, see Tables 1 and 3.

2.9. Bioassay

Human hepatoma Bel-7402 cells, human gastric cancer BGC-823 cells, human cervical carcinoma HeLa cells and human leukemia HL-60 cells were maintained in RPMI 1640 medium (GIBCO/BRL, Maryland, USA) supplemented with 10% (v/v) fetal bovine serum and cultured in 96-well microtiter plates for the assay. Appropriate dilutions (10^{-3} to 10^2 μmol/l) of the test compounds were added to the cultures. After incubation at 37 °C, 5% CO_2 for 72 h, the survival rates of the cancer cells were evaluated by the MTT method [20,21]. The activity was shown as the IC_{50} value, which is the concentration (μmol/l) of test compound to give 50% inhibition of cell growth. Results were expressed as the mean value of triplicate determinations. Taxol was selected as the reference substance.

Table 2
^{1}H NMR spectral data of compounds **3**, **4** and **11**–**13** (500 MHz, in DMSO-d_6)[a]

H	**3**	**4**	**11**	**12**	**13**
1	1.40	1.48	3.57brd(7.5)	1.37	1.43
	1.36	1.20		1.37	1.34
2	1.43	2.12	1.69	1.57	1.44
	1.30	1.72	1.69	1.35	1.31
3	3.85brs	3.31brs	3.95brs	3.90brs	3.88brs
4	2.11	1.68	1.80	1.80	1.72
	1.71	1.46	1.44	1.18	1.31
5	1.76	1.42	2.00	1.61	1.77
6	1.78	1.75	1.73	1.75	1.72
	1.35	1.35	1.42	1.15	1.32
7	3.80brs	3.80brs	3.83	1.70	3.78brs
				1.05	
8	1.55	1.56	1.60	1.39	1.49
9	1.57	1.57	1.48	1.65	1.59
11	1.32	1.32	1.21	1.33	1.33
	1.12	1.10	1.14	1.13	1.12
12	1.39	1.40	1.36	1.91	1.58
	1.33	1.32	1.29	1.59	1.52
15	1.62	1.65	2.11	1.87	2.07brs
	1.30	1.30	1.71	1.61	2.05brs
16	2.08	2.08	2.07	4.11brs	4.21
	1.65	1.65	1.69		
17	2.46	2.45	2.44	2.20d (5.0)	2.25d (5.5)
18	0.62 (3H, s)	0.61 (3H, s)	0.62 (3H, s)	0.55 (3H, s)	0.61 (3H, s)
19	0.89 (3H, s)	0.82 (3H, s)	0.94 (3H, s)	0.85 (3H, s)	0.90 (3H, s)
21	7.52d (2.0)	7.51d (2.0)	7.50d (2.0)	7.52d (2.0)	7.54d (2.5)
22	7.95dd (9.5, 2.0)	7.94dd (9.5, 2.0)	7.93dd (10.0, 2.0)	7.82dd (9.5, 2.0)	7.84dd (9.5, 2.5)
23	6.28d (9.5)	6.27d (9.5)	6.26d (10.0)	6.32d (9.5)	6.32d (9.5)
1-OH			4.82d (7.5)		
3-OH	4.21brs	4.46d (4.0)	5.14d (5.0)	4.19d (3.0)	4.20brs
7-OH	5.57d (4.0)	5.63d (4.0)	5.58d (4.5)		5.58d (3.0)
14-OH	5.54s	5.55s	5.56s	4.29s	5.62s
16-OH				4.87d (5.0)	4.90brs

[a] Those peaks whose multiplicity was not designated are multiplets (m).

3. Results

Twenty strains of filamentous fungi and four species of bacteria were screened for their capabilities to metabolize bufalin (**1**). TLC and HPLC analyses suggested that **1** could be actively metabolized by *Mucor* (*M. polymorphosporus* AS 3.3443, *M. spinosus* AS 3.2450, *M. spinosus* AS 3.3450, *M. subtilissimus* AS 3.2454), *Alternaria* (*A. alternata* AS 3.4578, *A. alternata* AS 3.577, *A. longipes* AS 3.2875), *Cunninghamella* (*C. blakesleana* AS 3.970, *C. elegans* AS 3.1207), *Absidia coerulea* AS 3.3389, and *Syncephalastrum racemosum* AS 3.264 strains. By spraying with 10% H_2SO_4 and heating at 120 °C, new fluorescent colorful spots could be observed in the thin-layer chromatograms of the biotransformed products. HPLC/DAD analysis also showed new peaks with on-line UV absorption maximum at 296 nm, which is characteristic for bufadienolides (Fig. 2). No similar spot or peak was observed in the culture control. The stability of bufalin in the culture medium was ensured by the substrate control. No new spot or peak was observed, either, suggesting that the formation of the products is due to the microorganisms.

Mucor spinosus AS 3.3450 was the most potent strain for bufalin and was therefore selected for preparative-scale biotransformation. A total amount of 800 mg of bufalin was used and 12 pure products isolated by repeated silica gel column chromatography and preparative reversed-phase liquid chromatography (Fig. 2). Based on high-resolution mass spectrometry and extensive NMR techniques, their structures were identified as 7β-hydroxyl bufalin (**3**), 3-*epi*-7β-hydroxyl bufalin (**4**), 1β-hydroxyl bufalin (**5**) [3], 15α-hydroxyl bufalin (**6**) [22], 15β-hydroxyl bufalin (**7**) [23], telocinobufagin (5-hydroxyl bufalin, **8**) [24], 11β-hydroxyl bufalin (**9**), 12β-hydroxyl bufalin (**10**) [25],

Table 3
^{1}H NMR spectral data of **6**, **7**, **9**, **10** and **14** (500 MHz, in DMSO-d_6)[a]

H	6	7	9	10	14
1	1.34	1.36	1.51	1.41	3.57brd(7.5)
	1.34	1.36	1.40	1.34	
2	1.35	1.51	1.44	1.47	1.80
	1.31	1.32	1.32	1.31	1.75
3	3.86brs	3.89brs	3.87brs	3.87brs	3.98brs
4	1.97	1.87	1.76	1.80	1.88
	1.16	1.15	1.15	1.18	1.38
5	1.65	1.64	1.91	1.69	1.91
6	1.75	1.65	1.78	1.75	1.69
	1.12	1.10	1.06	1.14	1.20
7	1.25	1.32	1.81	1.71	1.72
	1.00	1.06	1.17	1.12	1.13
8	1.46	1.47	1.61	1.45	1.46
9	2.39	1.67	1.53	1.58	1.45
11	1.77	1.63	3.87brs	1.42	1.29
	1.60	1.56		1.05	1.06
12	1.91	1.35	1.58	3.20brd(12.0)	3.16
	1.82	1.35	1.35		
15	4.02brs	4.38	1.92	1.81	1.69
			1.59	1.58	1.56
16	2.05	2.43	2.01	2.00	1.98
	1.38	1.40	1.50	1.60	1.59
17	2.66	2.27dd (6.0, 9.5)	2.40dd (6.0, 9.5)	2.99	2.97
18	0.56 (3H, s)	0.61 (3H, s)	0.81 (3H, s)	0.49 (3H, s)	0.48 (3H, s)
19	0.81 (3H, s)	0.84 (3H, s)	1.09 (3H, s)	0.84 (3H, s)	0.93 (3H, s)
21	7.50s	7.50d (2.0)	7.49d (2.0)	7.43d (2.0)	7.43s
22	7.67d (9.5)	7.93dd (10.0, 2.0)	7.90dd (10.0, 2.0)	7.84dd (10.0, 2.0)	7.84d (9.5)
23	6.28d (9.5)	6.26d (10.0)	6.25d (10.0)	6.27d (10.0)	6.28d (9.5)
1-OH					4.82d (7.5)
3-OH	4.09brs	4.14brs	4.10d (3.0)	4.14brs	5.14d (4.5)
11-OH			3.91d (3.5)		
12-OH				4.46brs	4.47d (5.0)
14-OH	4.00s	4.02s	4.01s	4.13s	4.18s
15-OH	4.70d (4.5)	4.65d (6.5)			

[a] Those peaks whose multiplicity was not designated are multiplets (m).

1β,7β-dihydroxyl bufalin (**11**), 16α-hydroxyl bufalin (**12**), 7β,16α-dihydroxyl bufalin (**13**), and 1β,12β-dihydroxyl bufalin (**14**), respectively. Among them, **3**, **10**, **12** and **13** were obtained as major products in 16.0, 11.0, 5.5 and 7.0% final yields, respectively. Metabolites **3**, **4**, **9**, **11**, **12**, **13** and **14** are novel bufalin derivatives hydroxylated at C-1β, C-7β, C-12β and C-16α positions. The ^{1}H and ^{13}C NMR spectral data of the new compounds were unambiguously assigned according to their DEPT, ^{1}H–^{1}H COSY, NOESY, HMQC and HMBC spectra [26]. The structures of **6**, **7** and **10** have been proposed in previous literatures but with no detailed spectral data [22,23,25]. Their NMR data were reported for the first time in this paper (Tables 1–3).

The molecular formula of **3** was established as $C_{24}H_{34}O_5$ according to the pseudo-molecular ion peak at *m/z* 403.2479 $[M + H]^+$ in the HR-MS spectrum, suggesting that **3** is a monohydroxylated product of bufalin (**1**). When compared to that of **1**, the ^{13}C NMR spectrum of **3** showed an additional oxygenated methine signal at δ 69.2. In addition, C-6 (δ 36.7) and C-8 (δ 45.9) shifted downfield by 10.2 and 4.7 ppm, respectively, suggesting that the additional hydroxyl group was introduced at C-7. The signal of H-7 (δ 3.80) appeared as an unusually broad peak with half-height width ($W_{1/2}$) of 24 Hz. Its large coupling constants result from the axial–axial couplings of H-7 with H-6β and H-8, indicating that 7-OH should be in the β-configuration. The NOE enhancement between H-7 and H-9 (δ 1.57) also supported the β-orientation of 7-OH. Accordingly, the signal of 14-OH (δ 5.54) significantly shifted downfield by 1.44 ppm, as a result of the intramolecular hydrogen bonding of 7β-OH and 14-OH. Based on the above evidence, compound **3** was identified as 7β-hydroxyl bufalin.

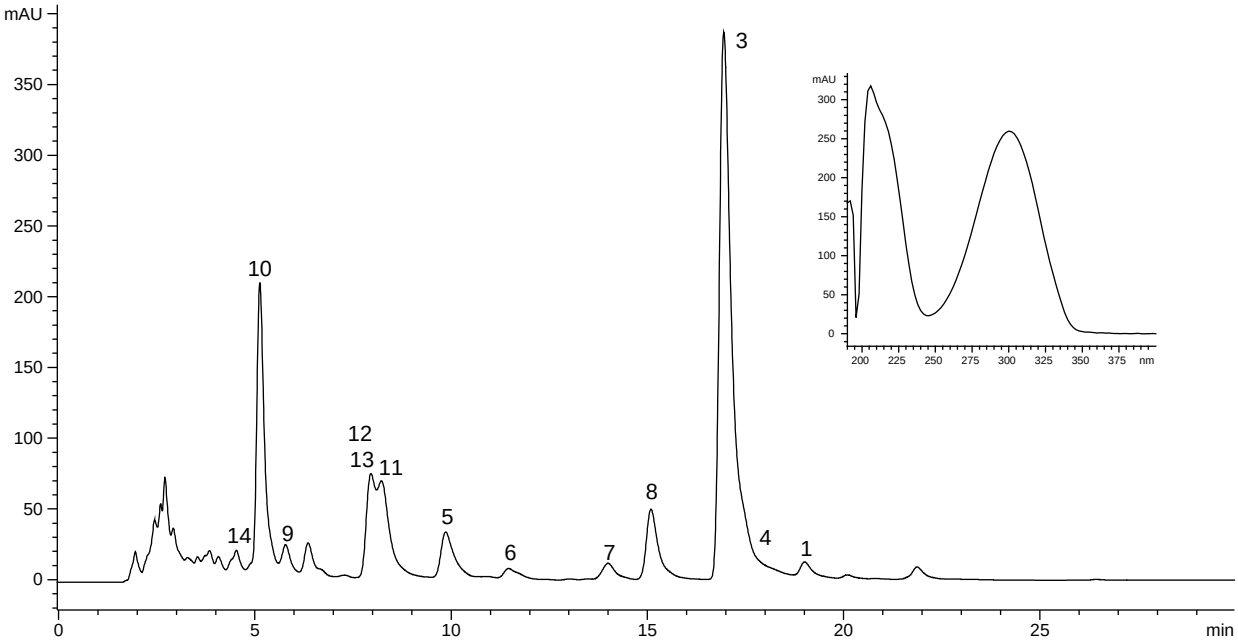


Fig. 2. HPLC profile of the biotransformed products from bufalin (**1**) by *Mucor spinosus* AS 3.3450, showing typical DAD on-line UV absorption spectrum of the products. For **1** and **3**–**14**, see Fig. 1.

Metabolite **4** was obtained and identified as a mixture with **3** in a molar ratio of 2:1. The two compounds showed very similar chromatographic behaviors, and were extremely difficult to be separated. Its ^{13}C NMR spectrum was essentially similar to that of **3** except the signals of ring-A carbons. An additional oxygenated CH signal appeared at δ 69.1, and both C-6 (δ 37.0) and C-8 (δ 46.1) significantly shifted downfield, consistent with **4** being a C-7β hydroxylated derivative of bufalin. The signal of H-3 shifted upfield to δ 3.31, and signals for ring A carbons are almost identical to those in 3-*epi*-desacetylcinobufagin [27], both suggesting that 3-OH in **4** should be in the α-configuration. Therefore, compound **4** was characterized as 3-*epi*-7β-hydroxyl bufalin. Its NMR spectral data were assigned by comparing with those of **3** and 3-*epi*-desacetylcinobufagin.

The HR-MS spectrum of **9** showed the $[M + H]^+$ ion peak at *m*/*z* 403.2475, suggesting the molecular formula of $C_{24}H_{34}O_5$. In the ^{13}C NMR spectrum, an additional oxygen-bearing tertiary signal appeared at δ65.3. The signal of C-12 (δ 48.1, t) shifted downfield by 8.1 ppm when compared with the corresponding signal in bufalin, indicating that C-11 in **9** was hydroxylated. The ^{1}H NMR signals of 18- and 19-methyl groups both shifted downfield due to their 1,3-*cis*-diaxial interactions with 11-OH [28], suggesting the axial position of 11-OH. The NOE enhancements of 11-OH with 18-CH_3 (δ 0.81) and 19-CH_3 (δ 1.09) were observed in the NOESY spectrum, which vigorously confirmed the β-configuration of 11-OH. In accordance, C-8 resonated at a higher field when compared to that in 11α-hydroxyl bufalin, as a result of *γ-gauche* effect of 11β-OH. H-11 appeared as a much narrower signal rather than the unusually broad peak in 5,11α-dihydroxyl bufalin ($W_{1/2} = 20$ Hz) [29] resulting from the axial–axial couplings of H-11β with H-9 and H-12α (Fig. 3). Therefore, the structure of **9** was identified as 11β-hydroxyl bufalin.

The HR-MS spectrum of **11** showed the $[M + H]^+$ ion peak at *m*/*z* 419.2432, suggesting the molecular formula of $C_{24}H_{34}O_6$. The ^{13}C NMR spectrum showed two additional oxygenated CH signals at δ 71.9 and δ 69.1, suggesting that **11** is a dihydroxylated product of bufalin (**1**). The signal of C-19 significantly shifted upfield to δ 18.6, suggesting that C-1 was hydroxylated. This was confirmed by the long-range coupling between 19-CH_3 (δ 0.94) and C-1 (δ 71.9) in the HMBC spectrum. The ^{1}H–^{1}H COSY spectrum showed the correlation between H-1 (δ 3.57) and H-3 (δ 3.95), which is a characteristic W-type ^4J long-range coupling, suggesting that both H-1 and H-3 are in the equatorial position [30]. In accordance, 3-OH resonated at a much lower field (δ 5.14), as a result of the intramolecular hydrogen bonding between 3-OH and 1-OH (Fig. 4). The NOE enhancements of 1-OH and 3-OH with H-5 (δ 2.00) also supported the β-configuration of 1-OH. The downfield shift of C-6 ($\Delta\delta$ +9.6) and C-8 ($\Delta\delta$ +5.0) in the ^{13}C NMR spectrum suggested that the other hydroxyl group in **11** was introduced at C-7. The signal of H-7 (δ 3.83) appeared as an unusually broad peak ($W_{1/2} = 24$ Hz), suggesting that 7-OH was in the β-configuration. This was further confirmed by the NOE enhancement between H-7 and H-9 (δ 1.48) in the NOESY spectrum. Therefore, the structure of **11** was identified as 1β,7β-dihydroxyl bufalin.

The HR-MS spectrum of **12** showed the $[M + H]^+$ ion peak at *m*/*z* 403.2479, suggesting the molecular formula of $C_{24}H_{34}O_5$. The ^{13}C NMR spectrum showed an additional CH signal at δ 76.6, and signals for A-, B- and C-ring

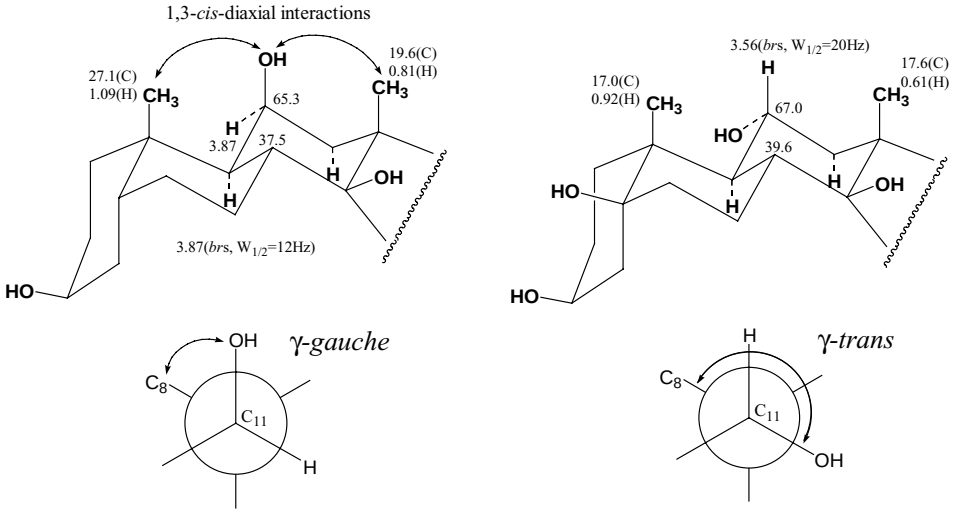


Fig. 3. Comparison of NMR data for 11β-hydroxyl bufalin (**9**, left, 500 MHz, in DMSO-d_6) and 5,11α-dihydroxyl bufalin (right, 600 MHz, in DMSO-d_6) [29].

carbons were essentially similar to those in bufalin. However, C-17 (δ 60.3) and C-15 (δ 40.8) shifted downfield by 10.2 and 8.8 ppm, respectively, suggesting that C-16 was hydroxylated in **12**. In the NOESY spectrum, H-16 (δ 4.11) showed NOE enhancements with H-22 (δ 7.82) and H-21 (δ 7.52), strongly indicating the α-configuration of 16-OH. In addition, H-17 (δ 2.20) exhibited a coupling constant of 5.0 Hz, similar to that of other 16α-OH bufadienolides. While the coupling constant of H-17 for 16β-OH analogues are 9.0–10.0 Hz, consistent with the dihedral angle of approximately 0° for H-16α and H-17 and the Karplus relationship [31]. Based on the above analysis, the structure of **12** was identified as 16α-hydroxyl bufalin.

The HR-MS spectrum of **13** showed the $[M + H]^+$ ion peak at *m/z* 419.2429, suggesting the molecular formula of $C_{24}H_{34}O_6$. The ^{13}C NMR spectrum showed two additional oxygen-bearing tertiary signals at δ 69.1 and δ 76.6, respectively, indicating that two hydroxyl groups were introduced at secondary carbons in **13**. When compared to corresponding signals of bufalin, C-6 and C-8 shifted downfield by 10.0 and 4.6 ppm, respectively, suggesting that C-7 was hydroxylated in **13**. The signal of H-7 (δ 3.78) appeared as an unusually broad peak ($W_{1/2} = 24$ Hz), indicating that 7-OH should be in the β-configuration. This also was supported by the NOE enhancements of H-7 with H-4α (δ 1.72) and H-9 (δ 1.59) observed in the NOESY spectrum (Fig. 5). Another significant difference of the ^{13}C NMR spectrum of **13** with that of bufalin was the downfield shift of C-17 ($\Delta\delta$ +10.1), suggesting that the other hydroxyl group in **13** was introduced at C-16. That was supported by the long-range coupling between H-17 (δ 2.25) and C-16 (δ 76.6). The NOE enhancements of H-16 (δ 4.21) with 18-CH_3 (δ 0.61), H-21 (δ 7.54) and H-22 (δ 7.84) in the NOESY spectrum, as well as the coupling constant of H-17 ($J = 5.5$ Hz), established the α-configuration of 16-OH. Based on the above evidence, compound **13** was characterized as 7β,16α-dihydroxyl bufalin.

The HR-MS spectrum of **14** showed the $[M + H]^+$ ion peak at *m/z* 419.2434, suggesting the molecular formula of $C_{24}H_{34}O_6$. The ^{13}C NMR spectrum showed two additional oxygen-bearing CH signals at δ 72.1 and δ 73.8, respectively, suggesting that **14** was a dihydroxylated product of bufalin. When compared to that of bufalin, C-18 (δ10.2) shifted upfield by 6.5 ppm, indicating that C-12 was hydroxylated in **14**. In accordance, the HMBC spectrum showed the long-range correlation between H-12 (δ 3.16) and C-18. The stereochemistry of 12-OH was established as β-configuration by comparing the carbon signals with those in 12β-hydroxyl bufalin (**10**). The significant upfield shift ($\Delta\delta$ −5.0) of C-19 (δ 18.7) suggested that C-1 in **14** also was hydroxylated. In the ^1H–^1H COSY spectrum, H-1 (δ 3.57) and H-3 (δ 3.98) showed W-type long-range coupling, suggesting that 1-OH was in the β-configuration. In accordance, 3-OH (δ 5.14) shifted downfield by 0.99 ppm because of the hydrogen bonding between 1-OH and 3-OH. Based on the above evidence, the structure of **14** was identified as 1β,12β-dihydroxyl bufalin.

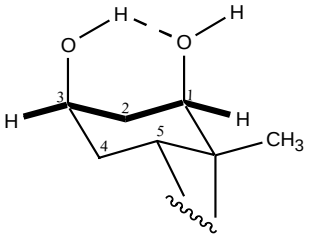


Fig. 4. Partial structure of 1β,7β-dihydroxyl bufalin (**11**), showing the W-type long-range coupling of H-1 and H-3 (bold line), and the hydrogen bonding of 1-OH and 3-OH (dotted line).

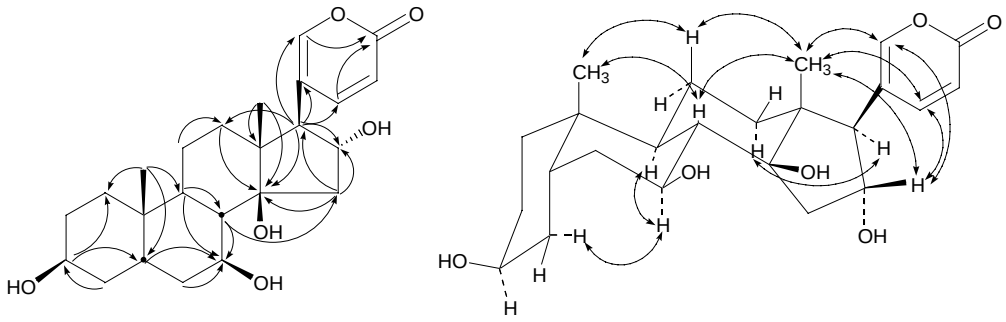


Fig. 5. Key HMBC (left) and NOESY (right) correlations of compound **13**.

Table 4
Cytotoxic activities of **1**–**30** against human cancer cell lines ($n = 3$)

Compound	IC_{50} (μmol/l)			
	Bel-7402[a]	BGC-823[b]	HeLa[c]	HL-60[d]
Taxol	3.4×10^{-1}	1.0	1.5×10^{-2}	4.4×10^{-4}
1	7.0×10^{-3}	4.5×10^{-2}	2.8×10^{-2}	5.0×10^{-3}
2	5.4×10^{-2}	6.5×10^{-2}	7.4×10^{-2}	3.4×10^{-2}
3	2.3×10^{-1}	2.0×10^{-1}	6.1×10^{-2}	5.7×10^{-2}
4	6.7×10^{-1}	6.3×10^{-1}	5.1×10^{-1}	5.3×10^{-1}
5	3.2×10^{-2}	2.4×10^{-2}	5.5×10^{-3}	3.5×10^{-2}
6	19.5	23.6	6.1	9.9
7	1.4	3.8	2.9	1.5
8	6.4×10^{-2}	8.8×10^{-2}	4.1×10^{-2}	5.3×10^{-2}
9	4.3×10^{-1}	3.6×10^{-1}	1.6×10^{-1}	2.8×10^{-1}
10	2.6×10^{-2}	3.1×10^{-2}	7.6×10^{-3}	3.4×10^{-2}
11	3.3	2.6	2.1	9.1×10^{-1}
12	6.7	7.6	3.8	1.4
13	37.2	57.1	43.4	24.5
14	4.0×10^{-1}	5.4×10^{-1}	2.5×10^{-1}	4.3×10^{-1}
15	7.7	8.1	4.8	5.2
16	32.9	43.1	51.5	28.6
17	7.7	8.9	4.6	6.1
18	58.8	58.8	>100	47.2
19	2.6×10^{-2}	3.0×10^{-2}	3.3×10^{-2}	2.6×10^{-2}
20	16.2	14.5	12.4	6.5
21	25.8	31.4	32.6	15.7
22	20.0	27.2	9.4	13.9
23	>100	4.7	1.8	2.6
24	5.5	5.4	4.4	4.4
25	3.7×10^{-1}	5.3×10^{-1}	1.9×10^{-1}	1.2×10^{-1}
26	56.4	63.8	42.4	27.0
27	4.6	4.2	1.1	3.3
28	62.5	–[e]	37.2	17.1
29	8.2	–[e]	4.0×10^{-1}	9.4×10^{-2}
30	6.1×10^{-1}	4.2×10^{-1}	2.2×10^{-1}	1.4×10^{-1}

[a] Human hepatoma cells.
[b] Human gastric cancer cells.
[c] Human cervical carcinoma cells.
[d] Human leukemia cells.
[e] Not determined.

The in vitro cytotoxic activities of **3**–**14**, together with 16 biotransformed products from cinobufagin (**15**–**30**) (Fig. 1), were determined with four human cancer cell lines by the MTT method [20,21]. The results are given in Table 4.

4. Discussion

Bufalin is an active substrate for microorganisms to produce a variety of metabolites. Different microbial strains showed different metabolic patterns on bufalin, while

strains from the same genus generally produced similar metabolites. It is noteworthy that most filamentous fungi produced 7β-hydroxyl bufalin (**3**), even as a major product for several strains (*Mucor*, *Aspergillus* and *Absidia*). *Pseudomonas aeruginosa* AS 1.860 was the only bacterium screened that could metabolize bufalin, and **3** was observed as the only product. The 12β-hydroxylated product **10** also was found to be a main product for several strains (*Mucor*, *Alternaria* and *Cunninghamella*). Thus 7β- and/or 12β-hydroxylation might be the predominant metabolic routes of bufalin by microorganisms. The microbial transformation of other related bufadienolides still needs to be investigated.

The biotransformation products **3**–**14** obtained in this study are bufalin derivatives hydroxylated at C-1β, C-5, C-7β, C-11β, C-12β, C-15α, C-15β or C-16α positions. All the oxyfunctionalities except 5-hydroxylation are novel for natural bufadienolides, and are obviously difficult to obtain by chemical means [1,2,32]. In previous studies [17,27], we obtained a series of new bufadienolides from cinobufagin by plant cell suspension cultures and microorganisms, including the novel 3-OH or 16-OH glucosylated, and 12β-hydroxylated products. In an effort to establish the structure–activity relationship of bufadienolides, the cytotoxic activities of these products were evaluated (Table 4).

Hydroxylation of bufalin at different sites could remarkably alter the cytotoxic activities. 1β-Hydroxyl bufalin (**5**) and 12β-hydroxyl bufalin (**10**) showed potent cytotoxicities comparable to bufalin. Both compounds are even more active against human gastric cancer BGC-823 cells and human cervical cancer HeLa cells with IC_{50} values of 10^{-8} to 10^{-9} mol/l. Compounds **3**, **8** and **9** showed a little weaker but still potent cytotoxicities than bufalin. However, hydroxylations at 15α-, 15β- or 16α-positions significantly reduced the activity, and the corresponding compounds **6**, **7**, **12** and **13** exhibited very weak or no cytotoxicities.

The 16-acetoxyl group is essentially important for the activities of cinobufagin derivatives. All deacetylated products (**15**–**18**, **24**, **26** and **28**) have very weak cytotoxicities, and glucosylation of 16-OH does not improve the activity (compounds **20**–**22**). However, the 3-OH glucosylated product (compound **19**), which was obtained as a major biotransformation product by *Catharanthus* cell suspension cultures, is two times more active than cinobufagin against all the four test cancer cell lines. The 12β-hydroxylation of cinobufagin is a popular reaction by filamentous fungi and slightly reduces the activities. Compound **17** is the only 12α-hydroxylated bufadienolide examined, and is about 10 times more active than its 12β-OH epimer **14**.

Bufalin derivatives are generally more active than corresponding cinobufagin analogues. Thus the 14β,15β-epoxy ring appears to reduce cytotoxicity. The results obtained in this study could lead to a preliminary structure–cytotoxic activity relationship of bufadienolides as illustrated in Fig. 6.

In conclusion, biotransformation of bufalin by *M. spinosus* yielded 12 products (**3**–**14**), including 7 new compounds with novel oxyfunctionalities at 1β-, 7β-, 11β-, 12β-, and 16α-positions. The in vitro cytotoxicities of 30 bufadienolides suggested that 3-OH glucosylation or hydroxylation at C-1β or C-12β positions might be promising reactions to obtain more polar bufadienolides with enhanced cytotoxic activities. The novel oxyfunctionalized derivatives of bufalin obtained in this study could provide new platforms for combinatorial synthesis [33–35] and other further investigations for the development of new bufadienolide antitumor drugs.

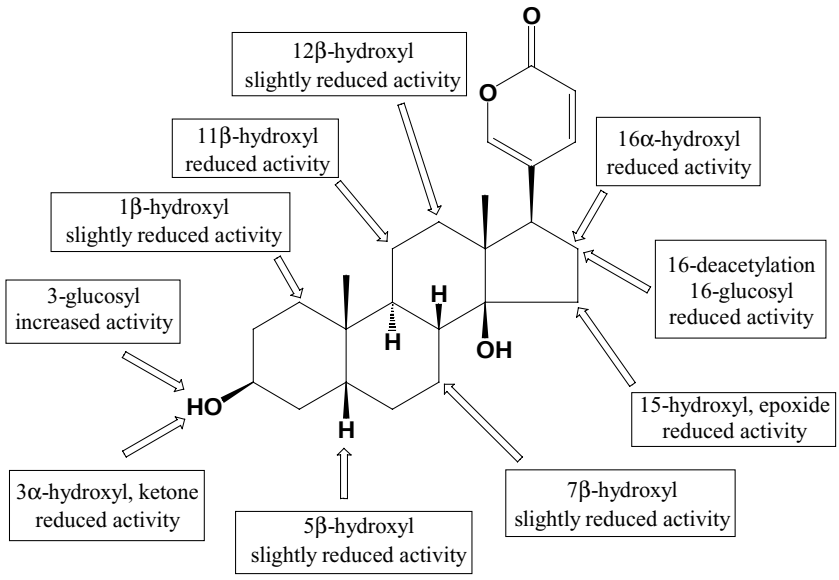


Fig. 6. Effects of structural modifications of cinobufagin and bufalin derivatives on growth inhibition of human cancer cell lines.

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